Ovarian function in ewes made hypogonadal with GnRH antagonist and stimulated with FSH in the presence or absence of low amplitude LH pulses

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Abstract

This study examined the effect of LH pulses, of similar amplitude and frequency to those found in the luteal phase, on the pattern of hormone secretion and follicle development in GnRH antagonist-suppressed ewes stimulated with exogenous FSH. This experiment was conducted on ewes with ovarian autotransplants in a continuous study. Follicle development was suppressed in 18 ewes by 3 weeks of GnRH antagonist treatment (50 µg/kg per 4 days s.c.), and was then stimulated by infusion of ovine (o)FSH (5 µg NIADDK-oFSH-16/h i.v.) for 3 days. In addition to FSH, 10 animals received pulses of LH (2.5 µg NIADDK-oLH-26 i.v.) every 4 h for the entire period of the FSH infusion. The follicle population was determined by daily ultrasound. Samples of ovarian and jugular venous blood were collected at 4-h intervals over the period of the FSH infusion and there were three periods of intensive blood sampling (15-min intervals for 2.5 h at 24, 48 and 72 h after the start of the FSH infusion) when the steroidogenic capacity of the follicles in all 18 ewes was tested around an LH challenge (2.5 µg i.v.).

GnRH antagonist treatment resulted in a 57% decrease in FSH concentrations and prevented ovarian follicle

development beyond 3 mm in diameter. Infusion of FSH resulted in a 60% increase in FSH concentrations and stimulated the development of large antral follicles and a coincident increase in ovarian androstenedione, inhibin and oestradiol secretion in both experimental groups. In the absence of 4-hourly LH pulses basal steroid secretion was negligible (<1 ng/min; $P < 0.001$). Daily LH challenges, however, revealed no difference in the steroidogenic capacity of the follicle population in either experimental group. Similarly, LH pulses had no effect on the growth rate and number of antral follicles stimulated by FSH infusion, or the pattern of ovarian inhibin secretion.

In conclusion, these results show that while FSH alone can stimulate the development of ovulatory sized follicles in ewes made hypogonadal with GnRH antagonist, physiological patterns of LH stimulation have no deleterious effects on FSH-stimulated follicle development and are essential for normal steroidogenesis.

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Introduction

It is generally accepted that follicle-stimulating hormone (FSH) is the key gonadotrophin responsible for the recruitment, growth and differentiation of follicles, whereas, while luteinizing hormone (LH) has a clear role in the acute control of follicular steroidogenesis and induction of ovulation, its role in folliculogenesis is unclear (Scaramuzzi *et al.* 1993). The apportioning of a pivotal role to FSH in the control of folliculogenesis was initially based on a large body of data reporting associations between circulating FSH concentrations and antral follicle development (reviewed by McNeilly *et al.* 1991), but more recently the

widespread availability of gonadotrophin-releasing hormone (GnRH) analogues has allowed this hypothesis to be tested more directly. These studies have shown that in a number of species, including sheep (Picton *et al.* 1990a), cow (Gong *et al.* 1996a,b), monkey (Yeoman *et al.* 1995) and human (Howles *et al.* 1994), the exogenous administration of highly purified FSH, but not LH, can stimulate the development of ovulatory-sized follicles in individuals in which hypogonadotrophic hypogonadism had been induced with potent GnRH agonists. Further, these FSH-dependent follicles are capable of ovulation of fertilizable oocytes and formation of normal corpora lutea following an ovulatory stimulus (Evans *et al.* 1994, Howles

et al. 1994, Gong *et al.* 1996b). This type of stimulatory regime has become the standard protocol for superovulation in human *in vitro* fertilization programmes (Howles *et al.* 1994).

Despite such clear evidence supporting a key role for FSH in antral follicle development, in large domestic ruminants both FSH and LH appear to be implicated in the growth of large oestrogenic or dominant follicles over a normal oestrous cycle. During the luteal phase, dominant follicles develop sequentially in a series of waves (sheep, Ginther *et al.* 1995, Souza *et al.* 1997a; cattle, Sirois & Fortune 1988) and although each wave is preceded by an increase in FSH (sheep, Ginther *et al.* 1995, Souza *et al.* 1997a,b; cattle, Adams *et al.* 1992) the life span of a dominant follicle can be altered by modulating the pulsatile pattern of LH secretion (Savio *et al.* 1993). Furthermore, following luteolysis, the final maturation and development of antral follicles to ovulation is dependent on an increase in the pulsatile secretion of LH (Baird & McNeilly 1981, McNatty *et al.* 1981). Studies using the GnRH agonist-induced hypogonadal model to examine the role of LH in folliculogenesis in sheep have produced equivocal results. While LH alone is unable to stimulate ovulatory follicle development in suppressed animals (Picton *et al.* 1990b) both LH antiserum (Picton & McNeilly 1991) and high amplitude/low frequency LH pulses can inhibit the stimulatory action of FSH on follicular growth (Picton *et al.* 1990b). The ability of LH to inhibit the action of FSH in stimulating follicle development is supported by a large body of evidence that LH can suppress the superovulatory response to FSH (sheep, Wright *et al.* 1981, Chupin *et al.* 1987; cattle, Murphy *et al.* 1984, Chupin *et al.* 1987; rat, Armstrong *et al.* 1989), and from these data a perception has developed that LH is detrimental to follicle development.

In addition to these direct effects on ovarian follicular development, LH is the primary drive for ovarian steroid secretion, and in sheep during the normal cycle pulsatile LH stimulation of the ovary results in pulsatile secretion of both thecal-derived androgens and membrana granulosa-derived oestrogens (Baird & Scaramuzzi 1976, Campbell *et al.* 1990). These ovarian steroids in turn have key autocrine and paracrine actions during follicular (Hillier 1987) and oocyte (Moor *et al.* 1980) development in addition to their major endocrine actions in the control of pituitary gonadotrophin release (Karsch *et al.* 1984, Baird *et al.* 1991), the responsiveness of the uterus to luteolytic signals (Lamming & Mann 1995), gamete and zygote transport (Harper 1988) and induction of behavioural oestrous (Baird & McNeilly 1981, Fabrenys *et al.* 1993). Given the key role of LH in ovarian steroidogenesis and its potential role in follicular development, it has always been somewhat surprising that stimulatory regimes that employ FSH alone in agonist-suppressed individuals are apparently so effective.

One possible explanation for this anomaly is that GnRH agonists only inhibit pulsatile, not basal LH secretion

(Picton *et al.* 1990a,b), and that basal LH is permissive for both FSH-stimulated follicle development and ovarian steroid secretion. This hypothesis is supported by the observation that LH antisera can inhibit FSH-stimulated follicle development in agonist-suppressed sheep (Picton & McNeilly 1991). In large domestic ruminants there are few data on the effect of agonist treatment on ovarian steroid secretion, but in the human it is clear that ovarian steroidogenesis is not depressed in GnRH agonist-suppressed women stimulated with highly purified urinary (Howles *et al.* 1994) or recombinant FSH (Devroey *et al.* 1994). Further, the fact that in species where overt signs of oestrus are evident, such as the pig and cow, heat is commonly observed in GnRH agonist-suppressed animals stimulated with pure FSH alone, suggests that ovarian oestradiol secretion is not depressed in these animals (pig, H M Picton, personal communication; cow, J G Gong and B K Campbell, unpublished observations). It has previously been shown that acute treatment of sheep with GnRH antagonist leads to inhibition of ovarian steroid secretion (Campbell *et al.* 1990) and there are reports that oestradiol secretion is minimal in GnRH antagonist-suppressed monkeys stimulated with pure FSH (Karnitis *et al.* 1994, Zelinski-Wooten *et al.* 1995). GnRH antagonist suppression may therefore represent a useful alternative model to study the modifying role of LH in FSH-stimulated follicle development.

The objective of the present study was to examine the effect of LH on the pattern of FSH-stimulated ovarian follicle development and ovarian steroid secretion in ewes in which hypogonadotrophic hypogonadism had been induced with a potent GnRH antagonist. This study utilized ewes with an ovarian autotransplant (Goding *et al.* 1967) as this model allows the morphological and endocrine responses of the ovary to be assessed simultaneously and repeatedly in conscious animals.

Materials and Methods

Experimental animals

Border Leicester Merino crossbred ewes ($n=18$; mean weight 65 ± 1 kg), with an autotransplanted ovary (Goding *et al.* 1967) were studied in the mid-breeding season (May). During the 3-week pretreatment with GnRH antagonist, the animals were kept outdoors and grazed normally with a ram fitted with a marking harness to verify the lack of oestrus behaviour (Radford *et al.* 1960). Prior to the experimental period, the animals were placed in metabolism crates in temperature-controlled rooms (20°C) under natural lighting at the CSIRO Division of Animal Production, Blacktown, NSW, Australia. The animals were fed a maintenance diet of 600 g daily of a pelleted ration of 60% lucerne and 40% oats and had been habituated to the housing conditions prior to the start of the experiment.

On the day prior to the start of blood sampling all animals, under local anaesthesia (Xylocaine, Astra Pharmaceuticals Pty Ltd, North Ryde, NSW, Australia), had a silastic cannula inserted into both jugular veins, as previously described (Campbell *et al.* 1994), in order to facilitate the collection of ovarian and jugular venous blood. In addition a third silastic cannula (0.8 mm inside diameter $\times$ 1.7 mm outside diameter; 602–175, Dow Corning, Midland, MI, USA) was inserted into the left jugular vein, caudal to the first cannula, for infusion of FSH. After cannulation, the animals were treated prophylactically with antibiotics (4 ml i.m., Hydropen, Bomac Laboratories, Castle Hill, NSW, Australia) and heparin (5000 IU i.v./12 h, Fisons Pharmaceuticals, Castle Hill, NSW, Australia).

Hormone solutions

The GnRH antagonist ([AcDNa¹,DCpa²,DTrp³,DArg⁶,DALa¹⁰]-GnRH; donated by the Salk Institute and Centre for Population Research, National Institute of Child Health and Human Development) was dissolved in 0.9% (w/v) sterile saline to a concentration of 1 mg/ml and administered s.c. (Campbell *et al.* 1990, 1992). The FSH used was NIADDK-ovine (o)FSH-S16 (bioactivity 20 times NIH-oFSH-S1 and LH contamination 0.04 times NIH-LH-S1/mg) whereas the LH was NIADDK-oLH-S26 (bioactivity 2.3 times NIH-oLH-S1 and FSH contamination <0.5% by weight). Both gonadotrophins were dissolved in 0.9% (w/v) sterile saline with 1% (v/v) normal sheep plasma. FSH was continuously infused with the aid of Harvard infusion pumps whereas LH was administered i.v. as a 2 ml bolus injection.

Experimental design

Ovarian suppression period In order to suppress ovarian function, all ewes were pretreated with GnRH antagonist (50 µg/kg s.c.) at intervals of 4–6 days for 3 weeks prior to the start of the experiment. Ewes were also treated over this period with intravaginal progestagen pessaries (medroxyprogesterone acetate, Repromap, Upjohn Pty Ltd, Rydalmere, NSW, Australia), which were changed at 10- to 12-day intervals, in order to more closely mimic the steroidal environment of a normal luteal phase. All ewes received cloprostenol (125 µg i.m., Estrumate, ICI plc, Macclesfield, Cheshire, UK) at the start of the pretreatment period to ensure the absence of corpora lutea. Samples of jugular venous blood were collected by venipuncture at each time of GnRH antagonist injection, for the determination of FSH, LH and progesterone concentrations.

Experimental period The animals were randomly divided into two groups. Group 1 ($n=8$) were treated with

FSH (5 µg/h) for 3 days without LH and Group 2 ($n=10$) were treated with FSH (5 µg/h) for 3 days with LH (2.5 µg administered as a bolus injection every 4 h). This dose of LH has been shown to induce an LH pulse of similar magnitude to that observed during a normal luteal phase (Baird *et al.* 1976, Wallace & McNeilly 1986, Whisnant *et al.* 1991). Ovarian (5 ml) and jugular (3 ml) venous blood samples were taken every 4 h over the experimental period and there were three periods of intensive blood sampling 24, 48 and 72 h after the start of FSH infusion. During intensive periods, both peripheral (2 ml) and ovarian venous (3 ml) blood samples were collected at 15-min intervals for 15 min before and 2 h after an LH challenge (2.5 µg i.v.) which was administered to both Groups 1 and 2. The blood was centrifuged at 4 °C and the plasma stored at -20 °C until analysis. To allow calculation of ovarian hormone secretion rates all collections of ovarian venous blood every 4 h, and every hour during the intensive sampling periods, were timed. The samples of ovarian venous blood taken every 4 h were collected 20–30 min after LH was administered to Group 2 ewes, as this is the time taken for maximal steroidogenic responses to LH stimulation (Campbell *et al.* 1990). After correcting for the haematocrit (Collett *et al.* 1973) and jugular contribution (inhibin only), the rates of ovarian secretion of oestradiol, androstenedione, progesterone and inhibin were calculated.

Ovarian scanning procedure

The ovarian follicle population was examined daily as previously described (Campbell *et al.* 1994). Briefly, the diameter of the antral cavity and position of all follicles greater than 2 mm in diameter in the medial/lateral, dorsal/ventral and cranial/caudal planes were determined using a combined sector real time ultrasound scanner with a sector 7.5 MHz transducer probe (Ausonics, Lane Cove, NSW, Australia).

Hormone assays

Plasma concentrations of LH, FSH, androstenedione (Campbell *et al.* 1990), immunoactive inhibin (McNeilly *et al.* 1989), oestradiol and progesterone (Campbell *et al.* 1994) were determined using previously described radioimmunoassays. The sensitivities of the assays for LH, FSH, androstenedione, inhibin, oestradiol and progesterone were 0.2 ng NIH-LH-S20/ml, 0.1 ng NIAMDD-FSH-RP1/ml, 20 pg androstenedione/ml, 35 pg 1–26 α -subunit porcine inhibin/ml, 15 pg oestradiol/ml and 200 pg progesterone/ml. The intra- and interassay coefficients of variation for LH, FSH, androstenedione, inhibin, oestradiol and progesterone were 4.3 and 5.4%, 1.7 and 2.8%, 6.2 and 9.1%, 5.2 and 10.5%, 6.4 and 10.2%, and 7.9 and 10.0% respectively.

Statistical analysis

Statistical analysis of hormonal profile data was performed by repeated measures analysis of variance (ANOVA) with log-transformed data partitioned on the basis of treatment and time. The characteristics of pulsatile LH secretion were determined using the Munro pulse analysis program (Zaristow Software, West Morham, Haddington, East Lothian, UK). The steroidogenic pulsatile response to an LH pulse was determined by grouping the data from individual profiles around the time of each LH injection and calculating basal secretion and pulse amplitude (defined as pulse height minus pulse nadir). In addition, the overall mean of LH, oestradiol and androstenedione secretion during the periods of intensive sampling were calculated. Comparisons between treatment groups on these determinants of pulsatile hormone secretion were made by repeated samples ANOVA. The effect of treatment on follicle numbers was determined by repeated sample ANOVA following $\sqrt{(x+0.5)}$ transformation.

Results

Peripheral hormone concentrations

The 3-week GnRH antagonist pretreatment resulted in a marked decrease ($P<0.01$) in both jugular venous plasma FSH concentrations (0.85 ± 0.07 ng FSH/ml before treatment to 0.48 ± 0.02 ng FSH/ml after pretreatment; mean $\pm$ S.E.M., $n=18$) and LH concentrations (0.55 ± 0.09 ng LH/ml before treatment to less than 0.2 ng LH/ml after pretreatment; mean $\pm$ S.E.M., $n=18$). Infusion of FSH restored FSH concentrations to pre-GnRH antagonist treatment values ($P<0.001$) within 8 h of the start of the infusion in both experimental groups (Fig. 1). Mean FSH concentrations remained at approximately 0.8 ng/ml for the remainder of the experimental period but did decline slightly in some animals (Fig. 2). Injection of LH pulses every 4 h had no effect on jugular FSH concentrations but, as expected, did result in a marked increase ($P<0.001$) in mean LH concentrations. Interestingly, within 24 h of the start of treatment, basal LH declined ($P<0.01$) and then remained stable for the rest of the experimental period at values similar to those detected before GnRH antagonist treatment. In contrast, mean LH concentrations in non-LH treated animals remained at the detection limit of the assay for the entire experimental period (Fig. 1). Analysis of the LH pulse profiles derived from the period of intensive blood sampling showed that injection of $2.5 \mu\text{g}$ LH resulted in a pulse with an amplitude of 3.8 ± 0.3 ng LH/ml (mean $\pm$ S.E.M., $n=18$) superimposed on a baseline concentration of 0.19 ± 0.04 ng LH/ml (mean $\pm$ S.E.M., $n=18$). There were no significant treatment effects on pulse amplitude or pulse nadir (data not shown) but LH pulse amplitude and mean

secretion declined with time ($P<0.05$) after the start of FSH infusion (Table 1).

Ovarian hormone secretion rates

Basal ovarian oestradiol and androstenedione secretion in ewes not frequently treated with LH remained below 1 ng/min for the entire experimental period (Fig. 1). In contrast, in Group 2 ewes (LH treated), basal androstenedione secretion increased ($P<0.001$) within 8 h of the start of FSH infusion and continued to increase ($P<0.01$) during the remainder of the experimental period. Basal oestradiol secretion was also stimulated ($P<0.01$) by LH treatment and although the rate of the increase from the start of FSH infusion was slower than that for androstenedione, taking approximately 24 h to increase markedly ($P<0.01$), values continued to increase ($P<0.001$) for the remainder of the experimental period. Ovarian immunoreactive inhibin secretion remained relatively stable for the first 24 h of the FSH infusion, then increased ($P<0.001$) in both groups, reaching a plateau around 64 h after the start of FSH infusion (Fig. 1). There were no differences in the level or pattern of inhibin secretion between Groups 1 and 2.

In contrast to basal steroid secretion, when the steroidogenic capacity of the follicle population in both groups was tested by LH challenges, there were no significant differences between the groups in the oestradiol or androstenedione pulse profiles obtained 24, 48 and 72 h after the start of FSH infusion (Fig. 1). Throughout the FSH infusion, however, there was a sequential increase in ovarian pulse nadir ($P<0.05$), pulse amplitude ($P<0.001$) and mean secretion ($P<0.001$) with time (24, 48 and 72 h) for both oestradiol and androstenedione (Fig. 1; Table 1).

Follicle development

Following 3 weeks of GnRH antagonist pretreatment there were no ovarian follicles greater than 3 mm in diameter and few follicles greater than 2 mm in diameter (Figs. 2 and 3). Infusion of FSH stimulated follicle growth and this was reflected in a sequential increase ($P<0.001$) in both follicle diameter (Figs 2 and 3a) and total number of follicles (Figs 2 and 3b). The increase in total follicle number was mainly composed of a sequential increase in the number of large (≥ 3.5 mm in diameter; $P<0.001$) follicles, whereas the number of small follicles increased ($P<0.05$) after 1 day of FSH infusion and then remained stable for the rest of the infusion period. Co-administration of pulses of LH every 4 h had no effect on the ability of FSH to stimulate follicle diameter (Fig. 3a) or follicle number (data not shown).

Follicle development and ovarian hormone secretion were temporally related and this is illustrated by Fig. 2 which shows follicle and hormonal data from an individual

Figure 1 The effect of a 3-day infusion of oFSH-16 ($5 \mu\text{g}/\text{h}$ i.v.) into ewes with ovarian autotransplants (pretreated for 3 weeks with GnRH antagonist to suppress endogenous gonadotrophins and hence large antral follicle development) that received no LH ($n=8$; ○, □) or LH ($2.5 \mu\text{g}$ oLH-S2 i.v.) every 4 h ($n=10$; ●, ■). Effects on (a) peripheral FSH (○, ●) and LH (□, ■) concentrations; (b) ovarian oestradiol secretion (○, ●); (c) ovarian androstenedione secretion (○, ●); and (d) ovarian inhibin secretion (○, ●) determined from samples of blood taken every 4 h. The inserts show the secretory response to a single test pulse of LH ($2.5 \mu\text{g}$ oLH-26 i.v., arrows) given to both groups of ewes 24, 48 and 72 h after the start of FSH infusion (the scale on the Y-axis is the same as the main figure). Values are means $\pm$ S.E.M. (S.E.M. for LH values are within the symbol).

Figure 2 The effect of a 3-day infusion of FSH (oFSH-16; 5 µg/h i.v.) in the presence of 4-hourly pulses of LH (2.5 µg oLH-26 i.v.) on the pattern of ovarian follicle development (top panel; each symbol represents the diameter of individual follicles identified by daily ultrasound) and jugular venous FSH concentrations (●), ovarian oestradiol (○), androstenedione (▲) and inhibin (△) secretion in an individual animal pretreated with GnRH antagonist to suppress endogenous gonadotrophins and hence large antral follicle development.

ewe from Group 2. The first day of FSH infusion stimulated the rapid recruitment of a number of small follicles and this was reflected primarily by an increase in ovarian androstenedione secretion. Follicle development continued during the second day of FSH treatment, but during this period androstenedione secretion remained stable and both ovarian inhibin and oestradiol secretion increased markedly while FSH concentrations tended to decline. The third day of FSH infusion saw a decline in the rate of large follicle development, the recruitment of a second cohort of small follicles and a marked increase in the ovarian secretion of oestradiol, androstenedione and inhibin.

Discussion

The findings described in this paper provide, for the first time, a longitudinal description of the pattern of follicle development and ovarian steroid secretion in GnRH antagonist-suppressed ewes in which follicle development has been stimulated by the infusion of physiological doses of FSH. The results show that while FSH alone can stimulate the development of preovulatory-sized follicles in hypogonadal sheep, these follicles, in the absence of pulsatile LH, secrete negligible quantities of androstenedione and oestradiol, thus confirming that GnRH antagonist treatment inhibits both immunoreactive and bioactive LH secretion. Furthermore, these data show that LH pulses, of similar amplitude and frequency to those observed during a normal luteal phase, restore ovarian steroid secretion without any effect on the pattern of FSH-stimulated follicle development.

The absence of ovarian steroid secretion in hypogonadal ewes stimulated with FSH alone not only emphasizes the key role that LH has in the control of ovarian steroidogenesis but also confirms that GnRH antagonist treatment inhibited both immunoreactive and bioactive LH secretion. GnRH antagonist treatment, while being as equally effective as GnRH agonist in suppressing FSH (50–60%; Picton *et al.* 1990a) appears to result in lower basal immunoreactive LH concentrations (values approximately 0.2 ng/ml) than GnRH agonist (values approximately 1 ng/ml; Picton *et al.* 1990b). It is clear, from the responses observed to daily LH challenges, that the follicle population retains the ability to elicit acute steroidogenic responses to LH, despite the absence of the normal patterns of episodic stimulation. This result shows very clearly that the level of ovarian steroid secretion depends not only on the presence of steroidogenic follicles in the ovary but also on the degree of LH drive. This is an important concept, because during reproductive phases when LH pulse frequency is low, such as during anoestrus (Souza *et al.* 1996) or during the mid- to late-luteal phase (Souza *et al.* 1997a), ovarian oestradiol secretion is low, despite the presence of large oestrogenic ovarian follicles. The lack of ovarian steroid secretion in GnRH antagonist-suppressed ewes stimulated with FSH emphasizes the unphysiological nature of the steroidal environment induced by such regimes and strongly suggests that physiological regimes of LH replacement would be needed if stimulatory regimes that employed GnRH antagonist in combination with recombinant FSH were used in a clinical context.

The second major finding of this work was that the addition of physiological-sized pulses of LH (Wallace & McNeilly 1986) had no effect, deleterious or otherwise, on the pattern of FSH-stimulated follicle development. This finding conflicts with a number of studies that have reported a deleterious effect of LH on FSH-stimulated follicle development (Chupin *et al.* 1987, Picton *et al.* 1990b). But it is consistent with the observation that

Table 1 Values of pulsatile LH and ovarian steroid secretion (mean $\pm$ S.E.M.) determined after a single bolus injection of LH (2.5 μ g LH-S26 i.v.), given 24, 48, 72 h after the start of FSH infusion (5 μ g/h i.v.), to ewes ($n=18$) with ovarian autotransplants pretreated with GnRH antagonist for 3 weeks to suppress ovarian follicle development. As there were no differences between Groups 1 and 2 for these values (see Fig. 1), data have been pooled across groups for this table

	Time (h)	Pulse amplitude	Basal secretion	Mean secretion
Hormone LH (ng/ml)	24	4.9 $\pm$ 0.5	0.2 $\pm$ 0.1	1.1 $\pm$ 0.1
	48	2.9 $\pm$ 0.3*	0.2 $\pm$ 0.1	0.6 $\pm$ 0.1*
	72	3.6 $\pm$ 0.5 $P<0.05$	0.2 $\pm$ 0.1 $P>0.05$	0.7 $\pm$ 0.1 $P<0.01$
Oestradiol (ng/min)	24	1.7 $\pm$ 0.3	0.3 $\pm$ 0.1	1.4 $\pm$ 0.2
	48	4.6 $\pm$ 0.8**	0.4 $\pm$ 0.1	3.0 $\pm$ 0.5**
	72	7.8 $\pm$ 1.5* $P<0.001$	0.8 $\pm$ 0.2* $P<0.05$	5.0 $\pm$ 1.0* $P<0.01$
Androstenedione (ng/min)	24	5.0 $\pm$ 1.3	0.3 $\pm$ 0.1	3.1 $\pm$ 0.7
	48	7.5 $\pm$ 1.4*	0.5 $\pm$ 0.1	4.8 $\pm$ 0.8**
	72	13.4 $\pm$ 2.5** $P<0.001$	0.6 $\pm$ 0.1 $P<0.05$	7.2 $\pm$ 1.3* $P<0.001$

P indicates overall significance of time effect. * $P<0.05$, ** $P<0.01$ from preceding time point (repeated samples ANOVA).

FSH-dependent follicle development in sheep (Webb & Gauld 1987, Souza *et al.* 1997a,b), and in a number of mono-ovulatory species (human, Baird 1983; cow, Webb *et al.* 1992), occurs during normal ovulatory cycles when LH secretion is pulsatile. The most likely explanation for the difference between the deleterious effects of LH pulses on FSH-stimulated follicle development reported previously and the lack of effect observed in the present experiment is the difference in LH pulse amplitude induced by injection of LH. Thus, it is likely that large amplitude pulses have a deleterious effect on the follicle population in a manner analogous to the fall in steroid secretion and induction of atresia in the antral follicle population that occurs following the preovulatory LH surge (Baird & McNeilly 1981). The recent finding that sheep follicular cells are exquisitely sensitive to LH in culture, and that continuous doses as low as 1 ng/ml can inhibit steroidogenesis (Campbell *et al.* 1996), supports this explanation.

In contrast to the results of the present study, and as detailed in the Introduction, in large domestic ruminants both FSH and LH appear to be implicated in the growth of large oestrogenic or dominant follicles over a normal oestrous cycle. However, data from our laboratory showing that FSH-dependent follicles can transfer their gonadotrophic dependence from FSH to LH, if it is delivered as a series of high frequency low amplitude pulses (Campbell *et al.* 1993), suggest that the involvement of LH in dominant follicle development is restricted to the terminal stages of follicular differentiation and may represent one of the mechanisms of follicle selection. The fact that follicles grow normally in the absence of LH, when follicular steroidogenesis is limited, is somewhat surprising given the widely held belief that steroids are important intrafollicular

modulators of follicle development (Hillier 1987). However, while it is possible that basal levels of LH are sufficient to maintain intrafollicular steroid concentrations, there have been no reports of steroid concentrations in the follicular fluid of FSH-stimulated GnRH antagonist-treated ewes.

The use of the ovarian autotransplant model in this experiment allowed a longitudinal description of the development of individual follicles and comparison of the pattern of follicle development with the precise pattern of hormone secretion in suppressed animals stimulated with FSH. The sequence of events observed following FSH stimulation was an initial increase in the number of small follicles, reflected hormonally by an initial increase in ovarian androstenedione secretion, followed by an increase in ovarian inhibin and oestradiol secretion as the recruited follicles continued to grow and mature under the influence of FSH. These follicular and ovarian secretory responses to FSH infusion are similar to those observed during FSH-stimulated follicle development in ewes immunized against inhibin (Campbell & Scaramuzzi 1995, Campbell *et al.* 1995) or following the FSH rebound in ewes treated with follicular fluid (Baird *et al.* 1990, Campbell *et al.* 1991).

In conclusion, these results show that while FSH alone can stimulate the development of ovulatory sized follicles in ewes made hypogonadal with GnRH antagonist, physiological patterns of LH stimulation have no deleterious effects on FSH-stimulated follicle development and are essential for normal steroidogenesis. Thus, in clinical or production contexts, LH replacement in GnRH antagonist-suppressed individuals is essential if a normal steroidal environment is to be maintained and LH will not antagonize the follicular response to FSH stimulation if physiological replacement regimes are used.

Figure 3 The effect of a 3-day infusion of FSH (oFSH-16; 5 µg/h i.v.) on (a) the mean diameter of all follicles detected by daily ultrasound in the ovaries of GnRH antagonist-suppressed ewes which received either no LH pulses ($n=8$) or 4-hourly pulses of LH (2.5 µg oLH-26 i.v., $n=10$); and (b) the number of small (<3.5 mm in diameter; solid bar) and large (≥3.5 mm in diameter; hatched bar) ovarian follicles detected by daily ultrasound per ewe (data from Groups 1 and 2 pooled). The summation of small and large indicate the total number of follicles. Values are means ± S.E.M. * $P<0.05$ or ** $P<0.01$ indicates significance of the difference from preceding time point for follicle diameter and number of small and large follicles, while † $P<0.05$ or †† $P<0.01$ indicates significance of difference from preceding time point for total number of follicles (repeated samples ANOVA).

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