Folliculogenesis and ovarian expression of mRNA encoding aromatase in anoestrous sheep after 5 days of glucose or glucosamine infusion or supplementary lupin feeding

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Improved nutrition increases ovulation rate in sheep and there is evidence that intra-ovarian pathways mediate responses to nutrition. An experiment was conducted to examine the effect of dietary energy on folliculogenesis. Anoestrous Merino ewes were fed a diet of wheat straw alone (control, $n = 5$), or wheat straw supplemented with lupins (500 g day^{-1} , $n = 5$). Other ewes were fed wheat straw and infused with glucose (50 mmol h^{-1} , $n = 5$) or with glucosamine (3.5 mmol h^{-1} , $n = 5$). Intravaginal progestagen sponges were inserted for 12 days, and nutritional treatments were started 5 days before sponge removal. At sponge removal, the ewes were injected with a regimen of GnRH pulses (500 ng every 4 h from 0 to 12 h; 250 ng every 2 h from 14 to 24 h; and 200 ng every 1 h from 25 to 36 h) to simulate normal follicular development. Thirty-six hours after sponge removal, the animals were killed and the ovaries were collected and stored at -80°C . The ovaries were sectioned serially every $10 \mu\text{m}$. Every 20th section was stained (to estimate number and diameter of follicles) and every 17–19th section was

probed by *in situ* hybridization for P₄₅₀ aromatase. Data were analysed using ANOVA and chi-squared tests. There was an effect of treatment ($P < 0.05$) on the number of follicles 2–3, 3–4 and 6–7 mm in diameter. Aromatase-positive follicles (1.6–7.9 mm) were detected in 31 follicles from 15 ewes across all four groups. In ten animals, the largest follicle was aromatase-positive. The diameters of aromatase-positive follicles were larger ($P = 0.004$) in lupin fed compared with glucose-infused ewes (4.9 ± 0.5 , 3.6 ± 0.7 , 5.3 ± 0.5 and $4.2 \pm 0.5 \text{ mm}$ for control, glucose-infused, lupin-fed and glucosamine-infused groups, respectively). Treatment did not affect the plasma concentration of FSH when compared with controls, indicating that the energy supplements were modifying recruited (2–3 mm and 3–4 mm) and selected follicles ($> 6 \text{ mm}$) directly. In conclusion, dietary energy can directly stimulate folliculogenesis in recruited and selected follicles, and this effect may be mediated by changes in systemic leptin concentrations and the hexosamine energy-sensing pathway in the follicle.

Introduction

Nutrition is a major factor determining the ovulation rate in domestic animals (Lindsay *et al.*, 1991) and the effect of nutrition is thought to be associated with alterations in the metabolic signals that mediate energy availability (Rhind and McNeilly, 1986; Rhind *et al.*, 1989; Downing, 1994). Ovulation rate is determined ultimately by the mechanism that controls selection of the ovulatory follicles from the follicular cohort. This mechanism is characterized by the complex relationship between intrafollicular growth factors and the hypothalamo–pituitary–ovarian feedback system during the final stages of follicular growth and development (Skinner *et al.*, 1987; Lobb and Dorrington,

1992; Scaramuzzi *et al.*, 1993; Monniaux *et al.*, 1994; Monget and Monniaux, 1995; Bao and Garverick, 1998).

An increase in the supply of energy can stimulate follicle development and increase ovulation rate in sheep. Ovulation rate is increased by the infusion of glucose into either the abomasum (Landau *et al.*, 1995) or the jugular vein (Downing *et al.*, 1995a), by the infusion of a mixture of branched chain amino acids into the jugular vein (Downing *et al.*, 1995b) and by dietary supplementation with lupin grain (Nottle *et al.*, 1988, 1990; Downing *et al.*, 1995c). All of these treatments also increase peripheral insulin concentrations (Downing *et al.*, 1995a,b,c), indicating that insulin-mediated glucose uptake, possibly by the ovary, modulates nutritional effects on ovulation rate. In support of this contention, the insulin-dependent glucose transporter

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(GLUT4) is present in both theca and granulosa cells of antral follicles in ewes (Williams *et al.*, 2001). An increase in the supply of energy substrates in this manner is thought to either promote the growth and development of follicles that would not normally ovulate or restore the ovulatory capacity of previously compromised follicles.

The mechanism that transduces this energetic input into a reproductive response in the ovary is not clear. The energy demands for folliculogenesis are minimal, so it is not the supply of energy *per se* that is responsible for this effect. A mechanism based on changes in hypothalamo–pituitary feedback is also unlikely because studies have been unable to establish a link between plasma FSH concentrations and ovulation rate (Findlay and Cumming, 1976; Bindon *et al.*, 1979; Cahill *et al.*, 1981; Driancourt *et al.*, 1988; Downing and Scaramuzzi, 1991). The direct action of a nutrient or metabolite on the follicle is a current theory and two potential mechanisms are leptin signalling in the follicle and nutrient sensing via the hexosamine biosynthetic pathway.

Leptin is a protein produced by adipose tissue and is involved in the regulation of appetite, energy expenditure and whole-body energy balance (Ingvarsten and Boisclair, 2001). Leptin also affects the reproductive system, acting either at the hypothalamus–pituitary axis, where it appears to help maintain adequate gonadotrophin secretion or by a direct action on the follicular granulosa cells (Spicer, 2001). Thus, in cultured bovine granulosa cells, leptin inhibits oestradiol production (Spicer and Francisco, 1997; B. K. Campbell, unpublished).

The hexosamine biosynthetic pathway can quantitatively assess metabolic status and act as a signalling pathway in some tissues (Wang *et al.*, 1998). Thus, tissues such as skeletal muscle and adipose tissue assess nutritional status so that they can respond to short-term variation in nutritional flux. Intracellular glucose enters the pathway as fructose 1,6-diphosphate and glucosamine is an intermediate in this pathway. One consequence of increased glucose flux through the hexosamine pathway in muscle is the increased expression of genes for leptin (Wang *et al.*, 1998) and two growth factors, fibroblast growth factor 2 (FGF2) (McClain *et al.*, 1992) and transforming growth factor α (TGF- α) (Daniels *et al.*, 1993; Roos *et al.*, 1996), which are potent regulators of granulosa cell proliferation and steroidogenesis (Gospodarowicz *et al.*, 1977; Gospodarowicz and Bialecki, 1979; Adashi *et al.*, 1987, 1988; Skinner and Coffey, 1988).

These potential roles of leptin as a nutritional mediator of follicular function and the hexosamine pathway as a follicular energy sensing mechanism have not been demonstrated. Therefore, the present study tested the hypotheses that: (i) short-term nutritional treatments that stimulate folliculogenesis will increase plasma leptin concentrations; and (ii) increased concentrations of glucose or glucosamine acting through the hexosamine biosynthetic pathway increase the number of follicles positive for aromatase gene expression.

Materials and Methods

Animals and treatments

Twenty-one adult anoestrous Merino ewes were treated for 12 days with medroxyprogesterone sponges (Repromap; Upjohn, Rydalmare). At the same time, the animals were allocated randomly to one of four treatment groups: (i) basal straw diet plus jugular i.v. saline infusion (control group, $n = 5$); (ii) basal straw diet plus 50 mmol glucose h^{-1} by jugular intravenous infusion ($n = 5$); (iii) basal straw diet plus 500 g lupin grain day^{-1} ($n = 6$); and (iv) basal straw diet plus 3.5 mmol glucosamine h^{-1} by jugular intravenous infusion ($n = 5$). The nutritional treatments lasted for 120 h and ended at the time of sponge removal. The day before the experiment started, bilateral jugular venous cannulae were inserted under xylocaine-induced local anaesthesia. One catheter was used exclusively for infusions and the other for collecting blood samples.

An artificial follicular phase to simulate normal follicular development was induced, using a regimen of treatment with GnRH. Starting at sponge removal, each ewe was treated with a regimen of intravenous pulses of GnRH (Sigma, St Louis, MO). The pulses were administered in three stages: 500 ng every 4 h from 0 to 12 h; 250 ng every 2 h from 14 to 24 h; and 200 ng every 1 h from 25 to 36 h. The animals were then killed with an overdose (5–6 g) of intravenous pentobarbital 156 h after the start of nutritional treatments and 36 h after sponge removal. Bilateral samples of ovarian venous blood (2–5 ml) were taken immediately and the ovaries were removed and stored at -80°C .

Collection of blood samples and hormone analysis

Concentrations of FSH, leptin, glucose and insulin were determined every 8 h starting 24 h before the commencement of nutritional treatments and continuing to the end of the experiment. Glucose was measured in whole blood using a Medisense® 2 glucose meter (Medisense Inc., Bedford, MA). The normal reading range was 1.1–33.3 mmol l^{-1} . Coefficients of variation (CV) for fresh human blood for low (2.2 ± 0.2 mmol l^{-1}) medium (4.5 ± 0.2 mmol l^{-1}) high–medium (11.1 ± 0.4 mmol l^{-1}) and high (14.2 ± 0.7 mmol l^{-1}) quality controls were 7.7, 4.1, 3.1 and 4.7%, respectively (Williams *et al.*, 2001). Blood was centrifuged at 1500 g for 10 min and the plasma was recovered and stored at -20°C . Total oestrogen concentrations were measured in ovarian venous blood, and FSH, insulin and leptin concentrations were measured in jugular venous blood using validated radioimmunoassays. Total plasma oestrogen was measured by radioimmunoassay based on the method of Webb *et al.* (1985). The total oestrogen concentration was measured in plasma after extraction of the samples with 4 ml ethyl acetate:hexane (3:2). The extract was redissolved in 0.10 ml gelatin–phosphate buffer and incubated overnight at 4°C with 0.10 ml antiserum (anti-Total Estrogens 1000T; ICN Biomedicals, CA) at a working dilution of 1:15 000.

Iodinated tracer (0.10 ml containing 10 000 d.p.m. 1,2,6,7-[H³]oestradiol) was added and the mixture incubated overnight at 4°C. Bound and free tracer were separated by incubating the assay mixture with 0.5 ml charcoal (0.5%) coated with dextran (0.05%) at 4°C and centrifuged at 3000 r.p.m. The major crossreactions of the antiserum are oestradiol (100%), oestrone (100%), oestrone-sulphate (60%), oestradiol-17 α (7%) and oestriol (415%). The limit of detection was 1.1 pg ml⁻¹. All the samples were measured in one assay for which the intra-assay CV was 4.7% at 7.4 pg ml⁻¹, 5.0% at 19 pg ml⁻¹ and 4.4% at 32 pg ml⁻¹. Plasma was assayed for FSH in duplicate by a double-antibody radioimmunoassay (Atkinson and Adams, 1988) using NIAMDD-oFSH-RP-1 (bio-potency 75 $\times$ NIH-FSH-S1) and NIADDK-anti-oFSH-1 serum. Plasma insulin concentrations were measured in duplicate by double-antibody radioimmunoassay (Tindal *et al.*, 1978). The samples were assayed as duplicate 200 μ l aliquots. A specific radioimmunoassay based on recombinant bovine leptin with an antiserum raised in an emu (*Dromaius novaehollandiae*) (Blache *et al.*, 2000) was used to measure leptin. Briefly, triplicates of standards and duplicates of unknown samples were incubated overnight at 4°C with diluted antiserum and normal emu serum and then incubated with labelled leptin for 48 h at 4°C. After incubation, diluted sheep anti-emu serum was added and the tubes were again incubated for 48 h at 4°C. Polyethylene glycol 6000 was added to the tubes before centrifugation. The limit of detection was 100 pg ml⁻¹ and the intra-assay coefficient of variation was 6.5% at a bound:free ratio of 65%.

The minimum detectable concentrations of FSH, insulin, leptin and oestradiol were 0.44 ng ml⁻¹, 4.5 μ U ml⁻¹, 0.2 mg ml⁻¹ and 1.0 pg ml⁻¹, respectively. The intra-assay CVs were 3.4, 7.3, 0.79 and 4.3% for FSH, insulin, leptin and oestradiol, respectively.

Number of follicles

The frozen ovaries of 21 animals (42 ovaries) were sectioned serially at 10 μ m intervals using a cryostat. The frozen sections were mounted on to glass slides (2–3 sections per slide) and fixed. The fixed slides were stored in 95% alcohol at 4°C. The number of follicles was determined by counting all microscopically visible follicles on every 20th slide stained with Harris's haematoxylin and eosin. The follicular diameter was calculated from the mean of two perpendicular diameters. The follicles were classified as follows: F < 1: follicles < 1 mm in diameter; F1: follicles 1–2 mm in diameter; F2: follicles > 2–3 mm in diameter; F3: follicles > 3–4 mm in diameter; F4: follicles > 4–5 mm in diameter; F5: follicles > 5–6 mm in diameter; F6: follicles > 6–7 mm in diameter; and F7: > 7 mm in diameter.

In situ hybridization

The expression of cytochrome P₄₅₀ aromatase was determined on dehydrated and fixed sections (10 μ m) of frozen ovaries (Wathes *et al.*, 1996; Leung *et al.*, 1999). The

sections were probed with [α ³⁵S]dATP (S) 1334; Amersham Pharmacia Biotech, Bucks) labelled DNA probes specific for P₄₅₀ aromatase (antisense; 45mer synthetic oligonucleotide). The probe sequences were based on the published cDNA sequence for aromatase (Hinshelwood *et al.*, 1993). The oligonucleotide 45mers was synthesized using the specific sequence 5'-TCA CCG GGT AGC CAT CGA TGA CAT CAT CCT CTA AGG CTT TGC GCA-3' (Leung *et al.*, 1999; Genbank accession number Z69249).

Established *in situ* hybridization procedures (Perks, 1994; Wathes *et al.*, 1996; Leung, 1997) were used with minor modifications. Briefly, the sections were impregnated with the reaction mixture containing labelled probe in hybridization buffer (100 000 c.p.m. per 100 μ l of hybridization buffer per slide), covered with a Parafilm coverslip, and incubated overnight at 42°C. After incubation, the sections were washed at room temperature in citrate buffer (SSC; 15 mmol sodium chloride l⁻¹, 15 mmol sodium citrate l⁻¹, pH 7.0, containing 0.2% (w/v) sodium thiosulphate-5 hydrate) in a shaking bath for 30 min followed by 1 h at 60°C. The slides were dehydrated in a gradient of ethanol, air dried and exposed to Kodak Biomax MR-1 film for 15 days. The sense probe was used as the negative control.

Photographic development

The manufacturer's (LM-1; Amersham Pharmacia Biotech) instructions were followed. Briefly, dried slides were dipped vertically into the emulsion for 5 s at 43°C and allowed to dry horizontally at room temperature and then on a metal tray pre-cooled with dry ice for 10 min each. The slides were then placed in a light-tight box with anhydrous silica gel in the base of the box and incubated for 3 weeks. After incubation, the slides were dipped into a developer (Phenisol; Ilford Limited, Ilford) for 5 min and then into a stop bath of 0.5% acetic acid (v/v) for 1 min. The developed slides were immersed in a fixative (47% (w/v), sodium thiosulphate pentahydrate) for 10 min and finally into distilled water for at least 10 min before counterstaining with Harris' haematoxylin and eosin.

Image analysis

After exposure, the photographic images of the ovarian sections were quantified for specific labelling using an image analysis system (Seescan, Cambridge) to measure the absorbance of specific areas (granulosa layer) identified on adjacent slides. The emulsion-coated slides were also used to confirm the cellular localization of the aromatase signal. The results from the autoradiographs were expressed as arbitrary units of absorbance with a linear range of 0.01–2.10. The non-specific counts (estimated from the sense probe) were subtracted from the total counts (estimated from the anti-sense probe) to produce a mean value for the specific hybridization in each follicle.

Statistical analyses

The concentrations of FSH, leptin, glucose and insulin were analysed for time and treatment effects using a split-

Table 1. Number (mean $\pm$ SEM) and size distribution of follicles in eight diameter classes of GnRH-treated anoestrous Merino ewes infused with glucose or glucosamine, or fed a supplement of lupin grain for 5 days

Follicle class (diameter, mm)	Control	Glucose	Lupin grain	Glucosamine
F0 (< 1)	36.7 $\pm$ 2.8	46.6 $\pm$ 9.1	42.7 $\pm$ 6.62	38.8 $\pm$ 10.3
F1 (1–2)	15.5 $\pm$ 2.2	24.4 $\pm$ 5.4	22.0 $\pm$ 5.9	15.8 $\pm$ 3.7
F2 (2–3)	4.1 $\pm$ 1.7 ^a	8.2 $\pm$ 3.4 ^b	2.7 $\pm$ 0.4 ^c	5.6 $\pm$ 1.9 ^a
F3 (3–4)	0.6 $\pm$ 0.3 ^a	2.6 $\pm$ 1.6 ^b	1.7 $\pm$ 0.2 ^b	2.0 $\pm$ 0.6 ^c
F4 (4–5)	1.3 $\pm$ 0.5	1.2 $\pm$ 0.2	0.5 $\pm$ 0.3	1.6 $\pm$ 0.5
F5 (5–6)	1.1 $\pm$ 0.3	0.6 $\pm$ 2.4	1.2 $\pm$ 0.2	0.6 $\pm$ 0.2
F6 (6–7)	0 ^a	0.2 $\pm$ 0.2 ^{a,b}	0.7 $\pm$ 0.2 ^b	0.2 $\pm$ 0.2 ^{a,b}
F7 (> 7)	0	0.2 $\pm$ 0.2	0	0

Values with different superscripts within rows differ significantly ($P < 0.05$).

plot ANOVA. Post-hoc differences between treatment groups were further examined using Tukey's test (SAS, 1995). Chi-squared tests were used to determine differences in the size distribution of follicles and to identify any differences in the number of follicles positive to P₄₅₀ aromatase.

Results

One animal in the lupin-fed group had an infected uterus and a persistent corpus luteum. This animal was excluded from the results and analyses.

Number and distribution of ovarian follicles

The mean number of follicles in each class decreased as average follicle size increased (Table 1). There was no effect of treatment on the number of follicles in the < 1 mm, 1–2 mm, 4–5 mm and 5–6 mm size classes. However, there were significant effects of treatment on follicles in the 2–3 mm, 3–4 mm and 6–7 mm classes ($P < 0.05$; Table 1).

There were effects of the nutritional supplements on both medium-sized (2–3 mm and 3–4 mm) and large (6–7 mm) follicles. Lupin-fed ewes had fewer follicles 2–3 mm in diameter ($P < 0.05$), whereas glucose-infused ewes had more follicles in this class ($P < 0.01$) compared with control and glucosamine-infused ewes. However, for follicles of 3–4 mm in diameter, all treated groups had more follicles than the control group. Follicles > 6 mm in diameter were found only in nutritionally supplemented ewes. The lupin-fed group had significantly more follicles of 6–7 mm in diameter than did the control group ($P < 0.05$).

Ovarian P₄₅₀ aromatase expression

Aromatase expression was confirmed in the granulosa cell layer (Fig. 1) and the concentration of mRNA in aromatase-positive follicles was not affected by treatment (Table 2). Nutritionally stimulated ewes tended to have more aromatase-positive follicles than did the control ewes but the differences were not significant (Table 2). However,

when the treated groups were pooled and the data re-analysed, the difference was significant (control 0.8 ± 0.37 and pooled-treated 1.92 ± 0.35 aromatase-positive follicles per ewe; $P < 0.05$).

The mean diameter of aromatase-positive follicles was affected by lupin and glucose treatments ($P < 0.01$; Table 2). The average diameter of aromatase-positive follicles was greatest in lupin-fed ewes and smallest in glucose-infused ewes (Table 2), although differences from average diameters of aromatase-positive follicles in control or glucosamine-infused groups were not significant.

Total oestrogen concentration in ovarian venous blood

The mean concentration of oestrogen was significantly greater ($P < 0.05$) in the ovarian venous blood from ovaries containing aromatase-positive follicles (46.8 ± 12.8 pg ml⁻¹) compared with that in ovarian venous blood from ovaries containing aromatase-negative follicles (19.6 ± 3.3 pg ml⁻¹). The mean concentrations of oestrogen in ovarian venous blood were not affected by treatment (Table 2).

Glucose concentration in jugular venous blood

The mean blood concentrations of glucose were increased by glucose infusion but were not affected by the other treatments (Table 3). The mean concentration of glucose in blood increased rapidly once the glucose infusion started and remained high for the duration of the infusion. At the end of infusion, the glucose concentrations in blood fell rapidly to pre-infusion values (Fig. 2).

Hormone concentration in jugular venous blood

The mean plasma concentrations of insulin were significantly increased by glucose infusion and feeding lupins (Table 3). However, the patterns of these increases differed (Fig. 3). In the glucose-infused group, the insulin concentration was increased significantly ($P < 0.05$) by 8 h after the beginning of infusion and remained high ($P < 0.05$) until the end of infusion at 120 h. In the lupin-fed group, the concen-

Fig. 1. (a,c,d) Antisense and (b,e,f) sense autoradiographic localization of mRNA for cytochrome P₄₅₀ aromatase in ovaries from GnRH-stimulated anoestrous Merino ewes. Note the intense localization of aromatase mRNA in the granulosa cells and its absence in theca cells. ar: aromatase; fa: follicular antrum; g: granulosa cells; t: theca cells. Scale bars represent (a,b) 2.5 μ m, (c–e) 10 μ m, and (f) 5 μ m.

tration of insulin was increased at 32 h, 80 h and from 96 to 120 h ($P < 0.05$) after the start of lupin feeding. Concentrations of insulin in the control and glucosamine-infused groups were not different from each other (Table 3; Fig. 3).

The mean plasma concentrations of leptin were affected by treatment with significantly higher concentrations in the glucose-infused and lupin-fed groups compared with the control and glucosamine-infused groups ($P < 0.05$; Table 3). The pattern of response differed in the two affected groups (Fig. 4). In glucose-infused ewes, leptin concentrations increased to a maximum at 16 h after the start of infusion

($P < 0.05$) and remained high for a further 32 h ($P < 0.05$), after which time they returned to control concentrations (Fig. 4). In the lupin-fed group, leptin concentrations increased more slowly to a maximum at 24 h after the start of feeding ($P < 0.05$) and remained high for another 72 h ($P < 0.05$) after which they returned to control concentrations (Fig. 4).

The mean plasma concentrations of FSH were not affected by any treatment when compared with the control ewes (Table 3). However, the mean plasma concentration of FSH in glucosamine-infused ewes was significantly lower

Table 2. Number and diameter of aromatase-positive follicles, aromatase (arbitrary units of absorbance) and the ovarian venous concentration of oestradiol of GnRH-treated anoestrous Merino ewes infused with glucose or glucosamine, or fed a supplement of lupin grain for 5 days

Treatment	Number of aromatase-positive follicles per ewe	Diameter of aromatase-positive follicles (mm)	Aromatase mRNA concentration (units of absorbance)	Total oestrogen concentration in ovarian venous blood (pg ml ⁻¹)
Control	0.8 ± 0.3	4.9 ± 0.5 ^{a,b}	0.41 ± 0.1	18.0 ± 3.9
Glucose infusion	2.0 ± 0.8	3.6 ± 0.7 ^a	0.32 ± 0.07	39.9 ± 18.9
Lupin fed	2.0 ± 0.0	5.3 ± 0.5 ^b	0.48 ± 0.04	37.2 ± 9.3
Glucosamine infusion	1.8 ± 0.6	4.2 ± 0.5 ^{a,b}	0.47 ± 0.07	40.0 ± 29.4

Values are mean ± SEM.

Values with different superscripts within columns differ significantly ($P < 0.05$).

Table 3. Blood concentration of glucose (mean ± SEM) and the plasma concentrations of insulin, FSH and leptin (mean ± SEM) of GnRH-treated anoestrous Merino ewes infused with glucose or glucosamine, or fed a supplement of lupin grain for 5 days

Treatment	Control	Glucose infused	Lupin fed	Glucosamine infused
Glucose (mmol l ⁻¹)	3.4 ± 0.04 ^a	4.3 ± 0.08 ^b	3.5 ± 0.04 ^a	3.3 ± 0.04 ^a
Insulin (μU ml ⁻¹)	12.7 ± 0.8 ^a	33.1 ± 2.1 ^b	20.9 ± 2.3 ^c	12.7 ± 1.2 ^a
Leptin (ng ml ⁻¹)	0.5 ± 0.01 ^a	0.7 ± 0.03 ^b	0.7 ± 0.03 ^b	0.5 ± 0.02 ^a
FSH (ng ml ⁻¹)	1.39 ± 0.05 ^{ab}	1.42 ± 0.04 ^{ab}	1.46 ± 0.06 ^a	1.29 ± 0.04 ^b

Values with different superscripts within rows differ significantly ($P < 0.05$).

Glucose, FSH and leptin showed an effect of day of treatment ($P < 0.05$).

Fig. 2. Mean blood concentration of glucose in anoestrous Merino ewes in which follicular growth was induced with a regimen of GnRH. Ewes were infused with saline (control, ◆; $n = 5$), glucose (■, 50 mmol h⁻¹; $n = 5$) or glucosamine (○, 3.5 mmol h⁻¹; $n = 5$) or fed a supplement of lupin grain (▲, 500 g day⁻¹; $n = 4$) for 5 days until the time of progesterone pessary withdrawal (arrow). Animals were killed 156 h after the start of infusion or feeding.

than it was in the lupin-fed ewes ($P < 0.05$; Table 3). There was a significant effect of time ($P < 0.05$) on plasma FSH concentrations (Fig. 5) and FSH declined significantly in all groups after the withdrawal of progestagen sponges at the end of the infusions.

Discussion

The regimen of GnRH injections used in the present study could simulate normal development of follicles in anoestrous ewes, and aromatase-positive follicles were

Fig. 3. The mean $\pm$ SEM plasma concentration of insulin in anoestrous Merino ewes in which follicular growth was induced with GnRH. The ewes were infused with saline (control, $\blacklozenge$; $n = 5$), glucose ($\blacksquare$, 50 mmol h^{-1} ; $n = 5$) or glucosamine ($\bigcirc$, 3.5 mmol h^{-1} ; $n = 5$) or fed a supplement of lupin grain ($\blacktriangle$, 500 g day^{-1} ; $n = 4$) for 5 days until the time of progesterone pessary withdrawal (arrow). Animals were killed 156 h after the start of infusion or feeding.

Fig. 4. The mean $\pm$ SEM plasma concentration of leptin in anoestrous Merino ewes in which follicular growth was induced with GnRH. The ewes were infused with saline (control, $\blacklozenge$; $n = 5$), glucose ($\blacksquare$, 50 mmol h^{-1} ; $n = 5$) or glucosamine ($\bigcirc$, 3.5 mmol h^{-1} ; $n = 5$) or fed a supplement of lupin grain ($\blacktriangle$, 500 g day^{-1} ; $n = 4$) for 5 days until the time of progesterone pessary withdrawal (arrow). Animals were killed 156 h after the start of infusion or feeding.

found in 15 of 20 ewes; in ten of these ewes, the largest follicle was also aromatase-positive. The results of the present study also show that the concentration of oestrogen in the ovarian vein draining an ovary containing an aromatase-positive follicle was significantly higher than the concentration of oestrogen in the ovarian vein draining an ovary containing only aromatase-negative follicles. These data are in agreement with other reports demonstrating that:

(i) the largest follicle is the principal source of ovarian venous oestradiol (Baird and Scaramuzzi, 1976); (ii) aromatase activity of granulosa cells and oestradiol concentration in follicular fluid are positively correlated in follicles $> 3.5 \text{ mm}$ in diameter (Monniaux, 1987); and (iii) aromatase activity is found principally in the granulosa cells of peri-ovulatory follicles in superovulated sheep (Lautinick *et al.*, 1994). The results of the present study show that the

Fig. 5. The mean $\pm$ SEM plasma concentration of follicle stimulating hormone (FSH) in anoestrous Merino ewes in which follicular growth was induced with GnRH. The ewes were infused with saline (control, $\blacklozenge$; $n = 5$), glucose ($\blacksquare$, 50 mmol h^{-1} ; $n = 5$) or glucosamine ($\circ$, 3.5 mmol h^{-1} ; $n = 5$) or fed a supplement of lupin grain ($\blacktriangle$, 500 g day^{-1} ; $n = 4$) for 5 days until the time of progesterone pessary withdrawal (arrow). Animals were killed 156 h after the start of infusion or feeding.

number of large, potentially ovulatory follicles was always greater than the number of aromatase-positive follicles. Therefore, the number of large follicles is not necessarily a reliable indicator of potential ovulation rate because large follicles must also be oestrogenic if they are going to ovulate (Webb *et al.*, 1989).

The following hypotheses were tested: (i) that short-term nutritional treatments that stimulate folliculogenesis will alter circulating plasma leptin concentrations; and (ii) that increased glucose or glucosamine acting through the hexosamine biosynthetic pathway will alter aromatase gene expression in the granulosa cells.

The results of the present study show that short-term nutritional supplementation can stimulate folliculogenesis and that the numbers of follicles 2–4 mm in diameter and > 6 mm were increased by nutritional supplementation. Follicles > 2.5 mm in diameter are normally considered to be capable of ovulating (Rhind and McNeilly, 1998). The data from the present study are in agreement with earlier results (Allen and Lamming, 1961), showing that ewes fed a high energy diet had increased numbers of follicles 2–3 mm in diameter. However, this is not a consistent observation and, in one study (Rhind and McNeilly, 1998), a high food intake increased the number of follicles 1.0–2.5 mm in diameter but not follicles > 2.5 mm in diameter. When ewes were fed lupin grain, the increase in ovulation rate was correlated with a greater number of follicles > 2 mm in diameter. Nottle *et al.* (1985) and Downing (1994) suggested that lupin grain supplementation decreased the number of follicles undergoing atresia. Increased energy supply did not affect the early stages of follicular development, but did increase ovulation rate by preventing atresia in follicles 2–3 mm in diameter without affecting the

number of follicles > 3 mm in diameter (Haresign, 1981). Follicles > 6 mm in diameter were seen only in ewes given the nutritional supplement, and the diameter of the aromatase-positive follicles was increased by lupin feeding. These findings indicate that nutritional supplementation increases ovulation rate by preventing atresia in large dominant follicles.

The number of aromatase-positive follicles was not affected by the treatments. However, closer inspection of the data showed that the number of aromatase-positive follicles in the energy-supplemented animals was about twice the number in the control group. There have been consistent reports that nutritional supplementation increases the number of large follicles. For example, 600 or 1200 g of a mixture of 10% lupin grain in hammer milled oats hay for 14 weeks increased the number of oestrogenic follicles and the total ovarian content of oestradiol in growing follicles (Boukhliq *et al.*, 1996), and the increased ovulation rate observed when feeding grain supplements has been associated with a larger number of follicles > 4 mm in diameter (Howland *et al.*, 1966).

One of the objectives of the present study was to examine the potential role of the hexosamine pathway as a nutrient-signalling pathway in the follicle. There is considerable data to show that the hexosamine pathway is a nutrient-signalling pathway in muscle and adipose tissue. Glucosamine is present in the follicular fluid of small and medium-sized follicles, but not in that of large follicles of buffalo (Boushehri *et al.*, 1996). In the ovary, eCG stimulates the rapid uptake of glucosamine by the zona pellucida and increases its concentration in follicular fluid from large follicles, whereas hCG enhances glucosamine uptake by the theca and granulosa of preovulatory follicles (Fowler

and Guttridge, 1987). Although these data show that glucosamine is present and active in the follicle, it is not directly concerned with its potential nutrient signalling role. The results of the present study indicate that glucosamine infusion stimulates folliculogenesis, as it increased the number of follicles of between 3 and 4 mm in diameter and, in this respect, mimicked the effect of glucose. Glucosamine infusion also tended to increase the number of aromatase-positive follicles and the number of follicles > 6 mm in diameter. Thus, glucosamine may be a nutrient-signalling pathway in the follicle, although further studies are required to confirm this tentative finding.

In addition to stimulating folliculogenesis, the nutritional supplements had a range of effects on circulating hormones. Lupin supplementation and glucose infusion both increased the plasma concentrations of insulin, indicating increased cellular uptake of glucose by follicles using the insulin-mediated GLUT4 pathway (Williams *et al.*, 2001). The effects of insulin on follicular cells include: (i) enhancement of glucose and amino acid metabolism; (ii) stimulation of cell proliferation and growth; (iii) stimulation and inhibition of follicular steroid secretion; and (iv) modulation of gonadotrophin receptor function (Allen *et al.*, 1981; Savio *et al.*, 1981; Rein and Schomberg, 1982; Poretsky *et al.*, 1985; Veldhuis *et al.*, 1985; Davoren *et al.*, 1986; Downing *et al.*, 1999).

There is evidence that leptin provides a signal of nutritional status to the reproductive system, and is the long sought-after mediator of nutritional status. Leptin is produced in adipose tissue, which actively responds to nutritional and metabolic changes. Leptin can act as a metabolic signal to the reproductive system by direct actions on the follicle (Spicer and Francisco, 1997). There are leptin receptors in the human ovary (Greisen *et al.*, 2000) and leptin can attenuate insulin-induced steroidogenesis of granulosa cells directly without affecting cell proliferation (Spicer and Francisco, 1997).

Leptin secretion is subject to both long-term and short-term regulation. Glucose, pyruvate and insulin all act on rat adipocytes *in vitro* to stimulate release and to increase synthesis of leptin (Levy and Stevens, 2001). Leptin concentrations in sheep are not responsive to short-term (< 2 h) changes in blood glucose or insulin (Kauter *et al.*, 2000). The present results confirm that, in sheep, a long-term glucose infusion increased plasma leptin concentrations by 8 h. The lupin supplement also increased plasma leptin concentrations but this effect was not observed until 24 h after the start of feeding. The results of the present study reveal some interesting temporal relationships among leptin, insulin and glucose. The leptin concentration was increased after either glucose infusion (increased by 8 h) or lupin feeding (increased by 24 h), but the increased concentration of leptin was not maintained despite the continued high concentration of glucose or insulin. Rats on high-fat diets may become resistant to leptin (Walder *et al.*, 1999; El-Haschimi *et al.*, 2000; Lin *et al.*, 2000, 2001) and a similar mechanism in sheep would explain the present

findings or indicate early signs of insulin resistance (Levy *et al.*, 2000; Chillard *et al.*, 2001). These results raise some questions about the interaction between insulin and glucose in the regulation of leptin secretion in ruminants.

In the present study, leptin had been at baseline concentrations for about 60 h before the ovaries were collected, indicating either a long-term effect of leptin on aromatase expression or, more probably, that a follicle, once selected, remains oestrogenic for at least 60 h (Campbell *et al.*, 1995; Souza *et al.*, 1996, 1997). The results of the present study in conjunction with other published information (Spicer, 2001) are consistent with the contention that leptin is a mediator of nutritional response on follicular function.

Glucosamine treatment did not alter the circulating concentrations of glucose, insulin or leptin. Nevertheless, glucosamine treatment did have effects on folliculogenesis and aromatase expression, indicating that its actions in follicular cells are intracellular. These findings are consistent with a proposed nutrient signalling role for glucosamine and the hexosamine pathway in the follicle, as occurs in skeletal muscle (Wang *et al.*, 1998; Ukropec *et al.*, 2001).

In conclusion, nutritional treatments have a direct stimulating action on folliculogenesis, which is independent of changes in circulating FSH. The effects were not the same across all three treatments and, furthermore, were observed at two different stages of folliculogenesis. The effect of lupin supplementation was mainly in large follicles > 6 mm in diameter, and this effect was not mimicked by infusion of either glucose or glucosamine. The effect of lupin grain supplementation is not completely mediated by increased insulin-mediated glucose uptake by the ovary. However, insulin-mediated glucose uptake did stimulate folliculogenesis, especially in medium-sized follicles 2–4 mm in diameter, an effect that was also observed in the glucosamine-infused ewes.

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