Comparative study between pony mares and ewes evaluating gonadotrophic response to administration of gonadotrophin-releasing hormone

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This study compared equine and ovine LH secretory responses to GnRH treatment. Dioestrous mares and ewes were challenged with continuous GnRH for 15 h. Mares that received constant GnRH ($110 \mu\text{g h}^{-1}$) had sustained LH secretion ($P < 0.01$), whereas LH concentrations in ewes treated with continuous GnRH ($25 \mu\text{g h}^{-1}$) initially increased, then declined and remained low, suggesting GnRH receptor desensitization or downregulation. In addition, progesterone-primed, ovariectomized mares and ewes were challenged with pulsatile or continuous GnRH for 5 days. Plasma LH concentrations were increased by day 5 in mares treated with pulsatile ($25 \mu\text{g pulse}^{-1} \text{h}^{-1}$) and continuous ($110 \mu\text{g h}^{-1}$) GnRH ($P < 0.01$). Furthermore, mean LH concentrations and time–response curves were not different. In contrast, ewes treated with continuous GnRH ($2.5 \mu\text{g h}^{-1}$) demonstrated LH secretory patterns indicative of GnRH receptor downregulation on day 1 of treatment. LH concentrations in ewes treated with pulsatile GnRH ($250 \text{ ng pulse}^{-1} \text{h}^{-1}$) did not differ from controls. In conclusion, pony mares responded continuously to GnRH treatment (pulsatile and continuous), whereas ewes treated with continuous GnRH experienced reduced LH secretion. These findings suggest a unique hypothalamic–pituitary axis in pony mares.

Introduction

GnRH and LH secretion has been described as rhythmically pulsatile in various species, including sheep (Clarke and Cummins, 1982) and primates (Dierschke *et al.*, 1970). In rodents, humans and sheep, exogenous administration of GnRH must be pulsatile to match endogenous GnRH release, providing normal physiological stimulation of LH (Santen and Bardin, 1973; Levine and Ramirez 1980; Moenter *et al.*, 1992). Conversely, continuous GnRH administration causes reduced LH release owing to GnRH receptor desensitization or downregulation (Nett *et al.*, 1981; Crowder *et al.*, 1986). Receptor desensitization is described as a loss of receptor binding owing to continuous ligand stimulation which may reflect physical or chemical changes within the receptor or second messenger system. These changes reduce its affinity for the ligand, thus preventing continued release of the target hormone (Dohlmán *et al.*, 1991; Lerrant *et al.*, 1995). In contrast, receptor downregulation is a loss of receptor binding owing to a reduction in the number of receptors, which causes a reduction in target hormone release (Clayton, 1989; Dohlmán *et al.*, 1991). The reduction in the number of receptors may be due, in part, to alterations in receptor synthesis (Lerrant *et al.*, 1995),

degradation (Chedrese *et al.*, 1994), recycling (Valiquette *et al.*, 1990), or post-receptor modifications (Dohlmán *et al.*, 1991). Although downregulation and desensitization of the GnRH receptor have been described in other species, they have not been described in horses. Furthermore, some studies evaluating gonadotrophin response to administration of GnRH suggest that mares may be resistant to GnRH receptor downregulation (Garcia and Ginther, 1975; Turner and Irvine, 1991). Therefore, we proposed to compare the LH response, as an indicator of receptor downregulation and desensitization, in sheep and horses, to continuous or pulsatile administration of GnRH.

Materials and Methods

Materials

Progesterone (4-pregnene-3,20-dione; Sigma P-1030, Sigma Chemical Company) was dissolved in benzyl alcohol (Fisher Scientific A396–500, Fisher Scientific, Pittsburgh, PA) and diluted to the desired concentration (25 mg ml^{-1}) in sesame oil (Sigma S-7131) (benzyl alcohol:sesame oil, 1:10). Progesterone implants were supplied by E. D. Plotka, Marshfield Medical Research Foundation, Marshfield, WI. Peripheral concentrations of progesterone ranged from 2.0 to 5.0 ng ml^{-1} within 1 week of implantation, consistent with previously published data

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concerning mid-luteal concentrations of progesterone in sheep (Quirk *et al.*, 1979).

Natural GnRH (BACHEM H-4005, purity HPLC > 99%, BACHEM BioScience Inc., King of Prussia, PA) was solubilized in sterile 0.9% (w/v) NaCl and diluted to the desired concentration in 0.9% saline. GnRH solutions were stored at -20°C .

Portable peristaltic pumps (Peristaltic Technologies, Inc., Mineoli, NY, Portable Pulsatile Drug Delivery Device, Model 1500; 5 ounces, $2.5\text{ cm} \times 2.75\text{ cm} \times 1\text{ cm}$) were validated in this laboratory to deliver $50\text{ }\mu\text{l}$ of fluid $\text{pulse}^{-1}\text{ h}^{-1}$ for 48 h. When set to constant infusion, pumps infused $50\text{ }\mu\text{l min}^{-1}$. A 9 V battery was required for each pump and needed replacement every 24 h. The life of a 9 V battery exceeded 24 h in the validation test, but the battery was replaced every 24 h during the experiment as a precaution.

Experiment 1 (mares)

During August, eight pony mares were selected from a resident herd. Mares had experienced normal oestrous cycles throughout the two preceding months, as monitored by daily teasing with a pony stallion, rectal palpation and rectal ultrasonography. All mares received 5 mg (i.m.) of prostaglandin $\text{F}_{2\alpha}$ (Lutalyse; UpJohn, Kalamazoo, MI) to synchronize oestrus. Mares were separated randomly into two groups 10–12 days after ovulation: group 1, control mares ($n = 4$); and group 2, GnRH-treated mares ($n = 4$). On the day of treatment, control mares were infused with isotonic saline continuously for 15 h while the treatment mares were infused with isotonic saline for the first 2 h and then with GnRH ($110\text{ }\mu\text{g h}^{-1}$) for the remaining 13 h. The dose of GnRH ($110\text{ }\mu\text{g}$) to be infused was based on studies by Becker and Johnson (1992), who infused $20\text{ }\mu\text{g}$ of GnRH in either a pulsatile or continuous fashion. We wished to challenge the equine pituitary and evaluate the LH secretory response to acute (13 h) administration of GnRH at supraphysiological concentrations. Automatic syringe pumps (Harvard Apparatus Co. Inc., South Natick, MA), calibrated to deliver 10 ml h^{-1} , were used. Mares were fitted with intravenous catheters (Angiocath; 14 gauge $\times$ 5.25 in) in left and right jugular veins and blood samples were collected from the contralateral jugular vein every 15 min.

Experiment 1 (ewes)

During December, eight cyclic Rambouillet ewes were selected from a residential flock. Normal oestrous cycles were observed in all ewes before the experiment. Ewes were selected to be 10–12 days after oestrus and were assigned randomly to two groups: group 1, control ewes ($n = 4$); and group 2, GnRH-treated ewes ($n = 4$). On the day of treatment, group 1 ewes were infused with isotonic saline continuously for 15 h, whereas Group 2 ewes were infused with isotonic saline for the first 2 h and then natural GnRH ($25\text{ }\mu\text{g h}^{-1}$) for 13 h. The dose of GnRH was determined based on studies by Nett *et al.* (1981). They administered GnRH at $2.5\text{ }\mu\text{g h}^{-1}$ and noted attenuation of LH secretion which suggested receptor desensitization. As in Expt 1 with mares, we wished to examine the LH response in ewes to acute administration of GnRH at

supraphysiological concentrations. All animals were fitted with intravenous jugular vein catheters in both right and left jugular veins and were housed in holding crates throughout the infusion period. Infusion pumps, infusion rates and infusion periods were similar to Expt 1 (mares). However, jugular blood samples were collected every 10 min rather than every 15 min.

Experiment 2 (mares)

Six adult ovariectomized pony mares were selected during May. Experiment 2 was designed as two replicates, separated by a 2 week recovery period, to maximize the number of mares in each group. To lower plasma LH to dioestrous concentrations before treatment, all mares received progesterone (75 mg per injection, i.m.) twice a day for 10 days before and throughout the treatment period. Five days before beginning treatment, subcutaneous tygon jugular vein catheters were installed. The catheters extended subcutaneously from the jugular vein to the prescapular region. Two days after catheter placement, girth straps carrying portable peristaltic pumps were fitted on all mares, and infusion lines from the pumps were connected to the subcutaneous tygon catheter at the exit point near the withers. The portable pumps were protected in a plastic housing so that the mares were free to move about their enclosure throughout the experiment except during sampling hours.

Mares were assigned randomly, in each replicate, to one of three groups: group 1, saline treated ($n = 2$); group 2, constant GnRH ($25\text{ }\mu\text{g h}^{-1}$, $n = 2$); and group 3, pulsatile GnRH ($25\text{ }\mu\text{g pulse}^{-1}\text{ h}^{-1}$, $n = 2$). The dose was significantly lower than that used in Expt 1 because we wished to evaluate the gonadotrophic response to chronic (5 days) administration of physiological concentrations of GnRH. On the morning of sampling period 1 (day 0; 1 day before onset of treatment), mares were fitted with intravenous sampling catheters in the jugular vein opposite the infusion jugular vein and blood samples were collected every 15 min for 8 h. Sampling catheters were left in place overnight for use the next day. On the next day (day 1 of treatment), peristaltic pumps were charged with GnRH solution for pulsatile infusion ($25\text{ }\mu\text{g}$ per $50\text{ }\mu\text{l pulse}^{-1}\text{ h}^{-1}$; group 3), constant GnRH solution ($25\text{ }\mu\text{g per } 3\text{ ml}$; group 2) or isotonic saline (group 1). Peristaltic pumps were validated in this laboratory to deliver $50\text{ }\mu\text{l fluid pulse}^{-1}\text{ h}^{-1}$ for 48 h. Group 1 mares (control) were randomly assigned to receive either constant saline or hourly pulses of saline. Treatment began on day 1 and blood samples were collected at 15 min intervals for 8 h as on day 0. After collection of the last sample, the sampling catheters were removed and all infusion reservoirs were refilled. Mares were monitored every 12 h on days 2, 3 and 4 to administer progesterone, collect blood samples (once per day), refill infusion reservoirs, replace the pump battery and for health management. On day 5 of treatment, mares were refitted with intravenous jugular vein catheters for collection of blood samples. Blood samples were collected every 15 min for 8 h, as on days 0 and 1. At the end of day 5, progesterone injections were terminated, sampling catheters were removed, portable pumps were turned off and girth straps removed. Subcutaneous infusion catheters were left in place throughout the 2 week recovery period and were

flushed daily with heparinized saline (Heparin (sodium salt) Sigma H-9399, Sigma Chemical Company, St Louis, MO). Catheter exit sites were examined daily and were treated with Furazolidone spray. At the end of the 2 week recovery period, administration of progesterone twice per day was resumed and maintained for 10 days before the onset of treatment and throughout the treatment period (days 1–5). The protocol described for replicate 1 was repeated in replicate 2.

Experiment 2 (ewes)

Nine adult, ovariectomized Rambouillet ewes were selected from a resident flock during December. Two weeks before the onset of treatment all ewes had subcutaneous progesterone implants surgically placed in the axillary region. Two days before sampling period 1, intravenous catheters were fitted in both jugular veins of all ewes. One jugular vein was used for sampling and the contralateral jugular vein was used for infusion of GnRH. Tygon catheter extensions were connected to jugular vein catheters (infusion catheter) and extended to portable peristaltic infusion pumps. Ewes were assigned randomly to three groups: group 1, control ($n = 3$); group 2, constant GnRH ($2.5 \mu\text{g h}^{-1}$, $n = 3$); and group 3, pulsatile GnRH ($250 \text{ ng pulse}^{-1} \text{ h}^{-1}$, $n = 3$). As for mares in Expt 2, GnRH concentrations (pulsatile and constant) were decreased from Expt 1 and the treatment period was prolonged (5 days) to evaluate the LH secretory response to chronic physiological stimulation. Blood samples were collected from all ewes 1 day before the onset of treatment (day 0), on day 1 of treatment and on day 5 of treatment for 8 h at 10 min intervals. Ewes were examined every 12 h on days 2, 3 and 4 and pump reservoirs were refilled, batteries replaced, and blood samples were collected each day.

Experiment 3 (mares)

Five weeks after the end of Expt 2, the same pony mares were used in Expt 3. As in Expt 2, all mares received progesterone (75 mg every 12 h, i.m.) for 10 days before day 1 of treatment and progesterone administration continued throughout the treatment period. Mares were assigned randomly to two groups: group 1, control (no treatment, $n = 4$); and group 2, constant GnRH ($100 \mu\text{g h}^{-1}$) ($n = 4$). Five days before treatment, group 2 mares had subcutaneous tygon catheters surgically placed, as in Expt 2. Two days later, group 2 mares were fitted with girth straps carrying portable peristaltic pumps. As in Expt 2, mares were sampled 1 day before the onset of treatment (day 0) and samples were collected at 15 min intervals for 8 h. Sampling protocol was repeated on day 1 of treatment and day 5 of treatment. In addition, blood samples were collected every 24 h on all days of treatment (days 1–5).

All animal work in all experiments was approved and regulated by the IACUC at the University of Florida.

Portable pump pack

Portable peristaltic pumps were housed in customized packs able to support the weight of the animal while allowing it to

move around freely while being treated. Plastic, electrical junction boxes (10 cm $\times$ 10 cm) were lined with closed cell foam padding to fit the pump device and reservoir. A plastic cover was fastened with four screws. The junction box was placed within a rubber inner tube (inner diameter 10 cm) and secured in place by inflating the inner tube. Duct tape was used to protect the inner tube from abrasion. Four holes were drilled into the cover of the junction box to prevent pump overheating. The distal end of the jugular catheter was connected via the 23 gauge needle hub to Silastic tubing (Dow Corp.) from the peristaltic pump. Infusion reservoirs were custom made from sealable plastic bags to hold approximately 45 ml. Tygon tubing leading out from the reservoir was sealed with silicon adhesive and connected to Silastic tubing used in the peristaltic pump. All tubing and reservoir bags were gas (NO) sterilized before use. The portable pack was fastened to a girth strap such that the pack was lateral to the spinal column, and a breast strap (5 cm nylon webbing) was used to prevent the girth strap from moving caudally. The girth strap was padded with 10 cm cotton webbing to minimize friction. Daily adjustments were made to maintain proper pack position.

Hormone assays of blood samples

Approximately 8–10 ml of blood was collected into heparinized tubes and stored at 2°C until plasma was separated by centrifugation at 3000 r.p.m. for 15 min. Plasma was stored at -20°C . LH content was measured by radioimmunoassay. Equine LH was analysed by radioimmunoassay as described by Thompson *et al.* (1983) using rabbit antiserum to ECG provided by D. Thompson, Louisiana State University, Baton Rouge, LA. Sheep anti-rabbit immunoglobins were supplied by Sigma Chemical Company and purified equine LH was provided by J. Roser, University of California, Davis, CA. The assay sensitivity, intra- and interassay coefficients of variation were 1.2 ng ml^{-1} , 4.5% and 4.6%, respectively. Ovine LH was measured with a polyclonal antibody (NIADDK-oLH-I-2) supplied by the National Hormone and Pituitary Program, Ogden BioServices Corp., Rockville, MD. The assay sensitivity, intra- and interassay coefficients of variation were 0.40 ng ml^{-1} , 7.2% and 4.6%, respectively. Ovine and equine progesterone concentrations were measured in plasma using a commercial solid phase radioimmunoassay kit (TKPG5) provided by Diagnostic Products Corp, Los Angeles, CA. The assay sensitivity, intra- and interassay coefficients of variation were 0.5 ng ml^{-1} , 4.3% and 4.5%, respectively.

Statistical analysis

Data from Expts 1, 2 and 3 were analysed using the Statistical Analysis System (SAS). A split-plot design with repeated measures was used in a least squares analysis of variance (ANOVA) to test for differences among treatment groups, and orthogonal contrasts of least square means were used to compare treatment groups and treatment group-by-day interactions. Tests for homogeneity of regression were performed on time-response curves representing treatment-by-day-by-sample interactions. In addition, LH concentrations in

blood samples collected each day were analysed by ANOVA and effects of treatment and treatment-by-day interactions were tested. Tests for homogeneity of regression among daily samples (time-response curves) were also performed.

Data from individual animals were analysed by cluster pulse analysis (Veldhuis and Johnson, 1986) for evidence of significant LH peaks. Each LH peak was defined by specific criteria: (1) an increase in LH concentration must have exceeded preceding LH values by a factor at least equal to the sensitivity of the assay; (2) the increase must have exceeded the mean LH concentrations plus two times the intra-assay coefficient of variation; and (3) each LH peak must consist of three consecutive points in time. Average peak interval, peak width and peak amplitude were measured and analysed using least square analysis of variance and orthogonal contrasts.

Results

Experiment 1 (mares)

Mean plasma progesterone concentrations were not different between group 1 mares ($8.0 \pm 0.6 \text{ ng ml}^{-1}$) and group 2 ($8.6 \pm 0.6 \text{ ng ml}^{-1}$) mares. The concentrations corresponded to mid-luteal phase concentrations as described by Sharp and Black (1973). However, group 2 mares (continuous GnRH) had significantly higher ($P < 0.009$) concentrations of plasma LH ($26.38 \pm 3.94 \text{ ng ml}^{-1}$ least square means $\pm$ SD) than group 1 mares (control; $10.00 \pm 2.97 \text{ ng ml}^{-1}$). Tests for homogeneity of regression indicated that the time-response curves representing each treatment were not parallel. This was due to an increase in plasma LH in group 2 (Fig. 1a), after the onset of GnRH treatment, and maintenance of plasma LH concentrations above baseline for the remainder of the infusion. Plasma LH concentrations in group 1 remained at baseline throughout the experiment.

Cluster analysis of LH peak number, interval, width and amplitude did not indicate differences in number of peaks, peak width or peak interval. However, LH peak amplitude was lower ($P < 0.01$) in mares treated with continuous GnRH ($15.75 \pm 5.00 \text{ ng ml}^{-1}$) than in control mares ($45.0 \pm 5.0 \text{ ng ml}^{-1}$; Table 1).

Experiment 1 (ewes)

Mean progesterone concentrations did not differ between ewes in group 1 ($2.8 \pm 1.6 \text{ ng ml}^{-1}$) and ewes in group 2 ($3.6 \pm 1.3 \text{ ng ml}^{-1}$). LH concentrations in group 2 ewes (continuous GnRH, $25 \mu\text{g h}^{-1}$) were significantly higher ($15.47 \pm 1.97 \text{ ng ml}^{-1}$; $P < 0.004$) than LH concentrations in group 1 ewes (continuous saline; $0.997 \pm 2.80 \text{ ng ml}^{-1}$). Plasma LH concentrations in group 2 were not maintained above baseline throughout the infusion but returned to baseline after 9 h of GnRH infusion (Fig. 1b). Time-response curves for each treatment were tested for homogeneity of regression and the curves were not parallel ($P < 0.01$). LH concentrations did not differ between groups for the first 2 h of sampling. However, 10 min after the onset of GnRH infusion, LH concentrations increased sharply in group 2 and continued to

Fig. 1. Time-response curves representing plasma LH concentrations (least square means $\pm$ SD) in mares (a) and ewes (b) treated with GnRH (Expt 1). (a) LH patterns in samples collected from mares every 15 min for 15 h. The curves representing control mares (∇) and mares treated with continuous GnRH ($\blacktriangledown$) were not parallel ($P < 0.01$). (b) LH patterns in samples collected from ewes every 10 min for 15 h. The curves representing control ewes (∇) and ewes treated with continuous GnRH ($\blacktriangledown$) were not parallel ($P < 0.01$). The arrow indicates the time at which GnRH infusion began.

rise for approximately 2.5 h. After 2.5 h of GnRH infusion, LH concentrations reached a plateau for 2.25 h and subsequently declined. Plasma concentrations of LH returned to near baseline concentrations in group 2 ewes within 6 h of reaching maximum concentrations. An apparent short-lived increase was observed in plasma concentrations of LH in group 2 approximately 9 h after onset of GnRH infusion. This was attributed to a brief rise and subsequent decline in plasma LH in two of the four ewes (Fig. 1b).

Cluster analysis of LH peak number, interval, width and amplitude did not indicate differences in LH peak interval or LH peak width. However, the number of LH peaks in group 2 ewes (1.75 ± 0.5) was significantly lower ($P < 0.01$) than in group 1 ewes (4.7 ± 0.6 ; Table 1). In addition, the LH peak amplitude was significantly greater ($P < 0.01$) in ewes treated with constant GnRH ($48.5 \pm 6.0 \text{ ng ml}^{-1}$) compared with control ewes ($2.10 \pm 7.0 \text{ ng ml}^{-1}$).

Table 1. Mean ($\pm$ SD) number of LH peaks in 15 h, peak interval, peak width and peak amplitude as determined by ANOVA

Group	Species	Number of LH peaks	LH peak interval (min)	LH peak width (min)	LH peak amplitude (ng ml ⁻¹)
Control	Mare	2.25 $\pm$ 0.75	155 $\pm$ 50.0	323.0 $\pm$ 131.0	45.0 $\pm$ 5.0
Constant GnRH	Mare	2.25 $\pm$ 0.75	315.8 $\pm$ 35.0	205.0 $\pm$ 113.0	15.75 $\pm$ 5.0*
Control	Ewe	4.67 $\pm$ 0.6	146 $\pm$ 24	106 $\pm$ 38	2.1 $\pm$ 7.0
Constant GnRH	Ewe	1.75 $\pm$ 0.6*	100 $\pm$ 29	206 $\pm$ 38	48.5 $\pm$ 6.0*

*Significantly different from control ($P < 0.01$).

Experiment 2 (mares)

Progesterone concentrations did not differ across replicates or between treatment groups or days of treatment. Plasma concentrations of progesterone were 7.54 ± 0.99 ng ml⁻¹ (replicate 1) and 9.15 ± 1.02 ng ml⁻¹ (replicate 2), similar to progesterone concentrations measured during dioestrus in Expt 1 (mares). Mean LH concentrations measured during the multiple sampling periods (days 0, 1 and 5) were significantly higher ($P < 0.04$) in group 3 (pulsatile GnRH) than in group 1 (control) and group 2 (constant GnRH). Furthermore, plasma LH concentrations were greater ($P < 0.01$) in group 3 (pulsatile GnRH) by day 5 of treatment than they were in group 2 (constant GnRH) or group 1 (control) (Fig. 2a).

Time-response curves representing LH concentrations measured on each of the multiple sampling days (days 0, 1 and 5) were tested for homogeneity of regression and were not parallel. Regression analysis of group 2 mares alone indicated that the day 5 LH time-response curve differed from that of day 0 and day 1 ($P < 0.001$).

Mean plasma concentrations of LH were significantly lower ($P < 0.02$) in control mares and constant GnRH mares than in pulsatile GnRH mares on days 4 and 5 of treatment (Fig. 3a). Furthermore, tests for homogeneity of regression indicated that the time-response curves (treatment-by-day) were not parallel.

Cluster analysis of LH peak number, interval, width and amplitude in all groups across all days revealed no differences in LH peak interval or width. However, there were significantly fewer peaks on day 5 in control mares (1.5 ± 0.76 ; $P < 0.01$) compared with mares treated with pulsatile GnRH (3.9 ± 0.76) and continuous GnRH (4.5 ± 0.76). The number of LH peaks

did not differ between constant GnRH mares and pulsatile GnRH mares on day 5. In addition, LH peak amplitude was significantly greater ($P < 0.01$) on day 5 in mares treated with

Fig. 2. Mean plasma LH concentrations (least square means $\pm$ SD) in progesterone-treated, ovariectomized mares (a,b) and ewes (c) treated with pulsatile (■) and constant (▨) GnRH (Expt 2 (a,c) and Expt 3 (b)), representing treatment-by-day interactions (□, control). Samples were collected every 15 min for 8 h. (a) Plasma LH collected from mares in Expt 2. ANOVA indicated that plasma LH concentrations were significantly ($*P < 0.01$) higher on day 5 in pulsatile GnRH-treated mares ($25 \mu\text{g pulse}^{-1} \text{h}^{-1}$) than in control and constant GnRH-treated mares ($25 \mu\text{g h}^{-1}$). (b) Plasma LH collected from mares in Expt 3. ANOVA indicated that plasma LH concentrations in constant GnRH-treated mares ($100 \mu\text{g h}^{-1}$) were higher ($P < 0.01$) on day 5 than in control mares. (c) Plasma LH collected from ewes in Expt 2. ANOVA indicated that plasma LH concentrations in constant GnRH-treated ewes ($2.5 \mu\text{g h}^{-1}$) were higher ($*P < 0.01$) on day 1 than controls or pulsatile GnRH-treated ewes.

Fig. 3. Time-response curves representing plasma LH collected from progesterone-treated, ovariectomized mares treated with pulsatile (▼) and constant (■) GnRH from day 0 to day 5 of treatment (treatment began on day 1; ○, control). (a) LH time trends measured in mares during Expt 2. The curve representing mares treated with pulsatile GnRH was not parallel to the curves representing control mares and mares treated with constant GnRH, owing to the increase ($*P < 0.02$) in LH secretion on day 4 and 5 in mares treated with pulsatile GnRH. (b) LH time trends measured in mares during Expt 3. The curve representing mares treated with constant GnRH was not parallel to the curve representing control mares, owing to the increase in LH concentrations ($*P < 0.01$) on day 4 and 5 in mares treated with continuous GnRH.

pulsatile GnRH (22.50 ± 30 ng ml⁻¹) compared with control mares (11.12 ± 1.4 ng ml⁻¹) and mares treated with constant GnRH (14.67 ± 0.93 ng ml⁻¹) (Table 2). LH secretory patterns from representative mares (Fig. 4b,d) suggest increased LH basal secretion on day 5 in pulsatile GnRH-treated mares. However, the LH secretory pattern remained episodic.

Experiment 2 (ewes)

LH concentrations were higher ($P < 0.01$) in group 2 (constant GnRH; 24.12 ± 2.63 ng ml⁻¹) than in group 3 ewes (4.02 ± 2.54 ng ml⁻¹) and control ewes (4.49 ± 2.61 ng ml⁻¹) on day 1 of treatment. Orthogonal contrasts also indicated that plasma LH concentrations increased from day 0 to day 1 in group 2 whereas there was no change in group 3 ($P < 0.01$). Furthermore, LH concentrations in group 2 decreased from day 1 to day 5, yet there was no change in group 3 LH concentrations (Fig. 2c).

Tests for homogeneity of regression indicated that the time-response curves representing treatment-by-day-by-sample interactions (LH concentrations measured during multiple sampling periods) were not parallel. These differences may be due primarily to the initial increase and subsequent decrease of plasma LH concentrations in group 2 ewes throughout day 1.

Cluster analysis indicated a significant reduction ($P < 0.02$) in mean number of LH peaks on days 1 and 5 in ewes treated with constant GnRH (3.30 ± 0.84 and 3.70 ± 0.84 , respectively) compared with day 0 (6.70 ± 0.84). There were no differences noted in mean LH peak interval; however, the peak width and amplitude were significantly greater ($P < 0.001$) on day 1 in ewes treated with constant GnRH (88.0 ± 9.2 min and 52.3 ± 5.2 ng ml⁻¹, respectively) compared with control ewes (54.41 ± 12.20 min and 7.54 ± 7.0 ng ml⁻¹) and pulsatile GnRH ewes (35.7 ± 9.2 min and 7.4 ± 5.2 ng ml⁻¹) (Table 2). LH secretory patterns from representative ewes (Fig. 4a) suggest a regular periodic release of LH in control animals. Continuous GnRH appears to disrupt this pattern, causing a marked increase in LH secretion followed by a decrease and lack of response to GnRH administration (Fig. 4e). In contrast, pulsatile GnRH appeared to maintain the periodic LH release (Fig. 4c).

Experiment 3 (mares)

Regression analysis of LH time trends from mares treated with continuous GnRH in Expt 2 indicated that on day 5 the time trends were different from those on day 0 and day 1. Thus, although mean concentrations of LH were different between pulsatile and continuous GnRH-treated mares, the data suggested an LH response that may have been minimal owing to the dose of GnRH administered. Therefore, in Expt 3, mares were treated with a dose of GnRH that was approximately four times more concentrated (100 µg of GnRH per 3 ml) than the dose used in Expt 2 (25 µg 3 ml⁻¹). Control animals were not treated with isotonic saline because of the improbability of stimulating an LH response by infusing NaCl indicated in Expt 2.

Mean LH concentrations measured during the multiple sampling periods were significantly greater ($P < 0.01$) on day 5 in group 2 (20.57 ± 3.98 ng ml⁻¹) than in group 1 (9.29 ± 4.50 ng ml⁻¹) (Fig. 2b). Time-response curves were analysed for homogeneity of regression and were not parallel. Time-response curves representing LH daily samples also were not parallel owing to the rise in LH concentrations in group 2 mares on days 4 and 5 of treatment (Fig. 3b).

There were no differences in mean number of LH peaks, peak interval or peak width between group 1 and group 2 across the

Table 2. Mean ($\pm$ SD) number of LH peaks in 8 h, peak interval, peak width and peak amplitude (ng ml^{-1}) as determined by ANOVA

Group	Species	Day	Number of LH peaks	LH peak interval (min)	LH peak width (min)	LH peak amplitude (ng ml^{-1})
Control	Mare	0	2.00 ± 0.76	181.81 ± 20.0	87.5 ± 12.0	12.75 ± 0.80
	Mare	1	3.00 ± 0.76	105.0 ± 16.0	55.75 ± 12.00	13.50 ± 0.80
	Mare	5	1.50 ± 0.76	69.1 ± 26.0	37.12 ± 18.80	11.12 ± 1.40
Pulsatile GnRH	Mare	0	3.25 ± 0.76	99.0 ± 21.0	51.3 ± 15.3	9.75 ± 1.00
	Mare	1	3.50 ± 0.76	123.9 ± 16.2	58.0 ± 12.0	8.75 ± 0.80
	Mare	5	3.90 ± 0.76	102.0 ± 26.4	44.67 ± 20.0	22.50 ± 1.30^a
Constant GnRH	Mare	0	4.75 ± 0.76	80.0 ± 16.1	49.0 ± 12.0	11.66 ± 0.92
	Mare	1	3.50 ± 0.76	48.41 ± 20.0	44.0 ± 12.0	13.00 ± 0.92
	Mare	5	4.50 ± 0.76	80.0 ± 16.2	60.0 ± 12.0	14.67 ± 0.93
Control	Ewe	0	5.0 ± 0.8	92.31 ± 14.0	48.33 ± 9.20	5.27 ± 5.20
	Ewe	1	4.4 ± 1.1	109.4 ± 18.3	54.41 ± 12.20	7.54 ± 7.00
	Ewe	5	4.33 ± 0.80	90.0 ± 14.0	60.0 ± 9.2	8.30 ± 5.20
Pulsatile GnRH	Ewe	0	6.70 ± 0.84	58.0 ± 14.0	33.3 ± 9.2	6.73 ± 5.20
	Ewe	1	5.7 ± 0.84	70.3 ± 14.0	35.67 ± 9.20	7.40 ± 5.20
	Ewe	5	4.7 ± 0.84	70.0 ± 14.0	46.7 ± 9.2	7.0 ± 5.2
Constant GnRH	Ewe	0	6.7 ± 0.84	63.7 ± 14.0	32.0 ± 9.2	5.60 ± 5.20
	Ewe	1	3.3 ± 0.84^d	73.3 ± 14.0	88.3 ± 9.2^c	52.3 ± 5.2^b
	Ewe	5	3.7 ± 0.84^e	80.0 ± 14.0	25.3 ± 9.2	4.26 ± 5.2

^aLH peak amplitude was significantly ($P < 0.01$) increased in mares treated with pulsatile GnRH by day 5 of treatment compared with control mares and mares treated with constant GnRH on day 5.

LH peak ^bamplitude and ^cwidth were significantly ($P < 0.001$) higher in ewes treated with constant GnRH on day 1 of treatment compared with those of control and pulsatile GnRH treated ewes on day 1.

Ewes treated with constant GnRH had significantly fewer LH peaks on ^dday 1 ($P < 0.01$) and ^eday 5 ($P < 0.02$) of treatment compared with day 0.

three sampling periods (days 0, 1 and 5). However, mean LH peak amplitude was significantly ($P < 0.01$) higher on day 5 in mares treated with continuous GnRH ($25.0 \pm 3.7 \text{ ng ml}^{-1}$) compared with control mares ($11.5 \pm 3.7 \text{ ng ml}^{-1}$) (Table 3). Furthermore, LH secretory patterns from representative mares treated with continuous GnRH suggested an increase in basal LH secretion that remained episodic (Fig. 4f).

Discussion

In mares, endogenous GnRH secretion has been described as 'irregularly episodic' and continuous during oestrus (Grubbaugh and Sharp, 1987; Alexander and Irvine, 1987). In addition, studies have characterized the endogenous secretion of GnRH in mares as being episodic, with secretory events occurring two to three times per 12 h during dioestrus and increasing in episode frequency during oestrus (Grubbaugh and Sharp, 1987; Irvine and Alexander, 1987). GnRH has also been described in mares as continuous throughout the oestrous cycle with intervening secretory events (Alexander and Irvine, 1987). Similarly, LH secretory patterns from representative animals (Fig. 4) suggest that LH secretion is not periodic in mares as it is in ewes. Alternatively, it may be described as having an irregular pattern that occurs regularly. These findings suggest that exogenous GnRH supplementation in mares may not be restricted to pulsatile administration as described in other mammals, including sheep, primates and humans.

The LH response to constant GnRH in Expt 1 demonstrated that pony mares responded to constant GnRH by maintaining

increased LH concentrations whereas ewes did not (Fig. 1a,b). Along with persistent high concentrations of LH, mares treated with continuous GnRH had significantly decreased LH amplitudes, suggesting that mares respond to constant GnRH with increased basal secretion of LH rather than with larger LH peaks. LH secretion in ewes treated with continuous GnRH was not maintained but rather demonstrated a pattern typical of receptor desensitization or downregulation. This pattern of LH secretion is similar to the endogenous LH pattern observed in ewes during the preovulatory LH surge (Karsch *et al.*, 1992). Simultaneous measurement of GnRH and LH in ewes by Moenter *et al.* (1990) indicated an extended GnRH surge accompanied by an LH surge that terminates before cessation of the GnRH surge (Moenter *et al.*, 1990). It has been speculated that termination of the preovulatory LH surge may be due, in part, to depletion of releasable LH from the pituitary and the downregulation of GnRH receptors (Crowder and Nett, 1984). Analysis of LH peaks in ewes treated with continuous GnRH indicated fewer LH peaks, suggesting the need for pulsatile GnRH administration. Furthermore, LH peak amplitude was increased in ewes treated with continuous GnRH, reflecting an initially large LH secretory response that diminished over time.

In Expt 2, mares treated with pulsatile GnRH had increased plasma LH concentrations on day 5 of treatment ($25 \mu\text{g pulse}^{-1}$); however, the same effect was not observed in mares treated with constant GnRH ($25 \mu\text{g h}^{-1}$). It was hypothesized that the dose of GnRH administered continuously ($25 \mu\text{g h}^{-1}$), derived from studies by Becker and Johnson (1992), may have been insufficient to elicit an LH response. Analysis of error

Fig. 4. LH secretory patterns of representative progesterone-treated, ovariectomized mares (■) and ewes (▼) from Expts 2 and 3. (a) Control ewe on day 5 (Expt 2). (b) Control mare on day 5 (Expt 2). (c) Pulsatile GnRH-treated ewe on day 5 (Expt 2). (d) Pulsatile GnRH-treated mare on day 5 (Expt 2). (e) Continuous GnRH-treated ewe on day 1 (Expt 2). (f) Continuous GnRH-treated mare on day 5 (Expt 3).

variance in group 2 mares (constant GnRH) showed high variance, suggesting a physiological response, although overall mean LH did not differ.

In Expt 3, plasma LH concentrations on day 5 were increased in mares treated with continuous GnRH ($110 \mu\text{g}$

h^{-1}). LH concentrations did not appear to differ between pulsatile GnRH mares in Expt 2 ($17.38 \pm 1.71 \text{ ng ml}^{-1}$) and the constant GnRH mares in Expt 3 ($20.57 \pm 3.98 \text{ ng ml}^{-1}$). Furthermore, LH peak amplitudes were increased in both groups on day 5 and neither pulsatile GnRH nor constant

Table 3. Mean ($\pm$ SD) number of LH peaks in 8 h, peak interval, peak width and peak amplitude as determined by ANOVA

Group	Species	Day	Number of LH peaks	LH peak interval (min)	LH peak width (min)	LH peak amplitude (ng ml ⁻¹)
Control	Mare	0	3.0 $\pm$ 1.3	93.0 $\pm$ 23.0	97.0 $\pm$ 33.0	9.10 $\pm$ 3.7
	Mare	1	3.3 $\pm$ 1.3	138.7 $\pm$ 31.0	92.0 $\pm$ 44.0	11.0 $\pm$ 4.0
	Mare	5	1.7 $\pm$ 1.3	142.4 $\pm$ 31.0	145.0 $\pm$ 33.0 ^a	11.5 $\pm$ 3.7
Constant GnRH	Mare	0	4.5 $\pm$ 1.3	63.0 $\pm$ 20.3	62.5 $\pm$ 28.0	10.2 $\pm$ 3.7
	Mare	1	4.0 $\pm$ 1.3	80.75 $\pm$ 20.30	35.2 $\pm$ 28.0	11.5 $\pm$ 3.7
	Mare	5	2.0 $\pm$ 1.3	103.7 $\pm$ 20.3	63.5 $\pm$ 28.0	25.0 $\pm$ 3.7 ^b

^aLH peak width was significantly ($P < 0.04$) higher in control mares on day 5 of treatment compared with mares treated with constant GnRH on day 5.

^bLH peak amplitude was significantly ($P < 0.01$) higher in mares treated with constant GnRH on day 5 of treatment compared with control mares on day 5.

GnRH affected the number of LH peaks. The lack of change in the number of LH peaks, considering the hourly pulses, suggests that the LH secretory response exceeded the time interval between pulses such that LH secretion increased after the first pulse and remained high through the subsequent pulses. These findings indicate that pulsatile GnRH (25 μ g pulse⁻¹ h⁻¹) and constant GnRH infusion (100 μ g h⁻¹) elicit similar LH secretory responses in progesterone-treated, ovariectomized mares. This supports the conclusions of a study with anoestrous mares in which pulsatile GnRH and constant GnRH infusion (administered by slow release implants) were equally effective in inducing gonadotrophin secretion, follicular growth, ovulation and formation of a corpus luteum (Turner and Irvine, 1991). However, Becker and Johnson (1992) administered pulsatile (20 μ g pulse⁻¹ h⁻¹) or continuous GnRH (20 μ g h⁻¹) to cyclic and anoestrous mares and concluded that continuous GnRH affected plasma concentrations of LH in a different way to pulsatile GnRH (Becker and Johnson, 1992). The apparent lack of stimulation of LH by continuous GnRH in Expt 2 (current study) and the study by Becker and Johnson suggest an inadequate GnRH dose. The larger dose of continuous GnRH administered in Expt 3 (110 μ g h⁻¹) resulted in a clear increase in LH.

All mares received progesterone throughout the experiment. The LH secretory response in mares receiving GnRH (pulsatile or continuous) was not significant until days 4 and 5 of treatment. In addition, there was a tendency for a reduction in the number of LH peaks by day 5 in control mares, which received no exogenous GnRH. These data suggest that progesterone treatment reduced the pituitary response to exogenous GnRH in treatment mares and may have suppressed the hypothalamic-pituitary axis in control mares. Repeated GnRH administration may have increased the number of pituitary GnRH receptors or the affinity of the receptors to overcome the negative effect of progesterone on the anterior pituitary. Studies in sheep and rats have shown that progesterone can reduce the amount of mRNA encoding GnRH receptor as well as the number of GnRH receptors (Wang *et al.*, 1988; Laws *et al.*, 1990). In addition, GnRH receptor upregulation has been demonstrated in rats treated with pulses of GnRH (Kaiser *et al.*, 1993). Therefore, it is plausible that a similar mechanism occurs in progesterone-treated, ovariectomized mares.

Plasma progesterone concentrations exceeded 4 ng ml⁻¹ in both dioestrous ewes (Expt 1) and progesterone-treated

ovariectomized ewes (control ewes in Expt 2). However, the number of LH peaks (frequency) tended to increase slightly (approximately 1 pulse per 1.5 h) in progesterone-treated ovariectomized ewes (control ewes in Expt 2) compared with previously published values (1 pulse per 3 h) measured in Suffolk ewes (Goodman and Karsch, 1980). The slight variation in number of pulses reported in our study may have been due, in part, to differences among breeds of sheep (Suffolk versus Rambouillet). However, emphasis was placed on the similar concentrations of progesterone between the dioestrous ewes and the progesterone-treated ovariectomized ewes measured in this study, indicating that the progesterone-treated ovariectomized ewes represented ewes during the luteal phase.

In Expt 2 (ewes), LH concentrations differed between ewes treated with continuous GnRH and controls; however, neither mean LH concentrations nor number of LH peaks differed between ewes treated with pulsatile GnRH (250 ng h⁻¹) and control ewes. Since the rate of GnRH pulse administration (1 pulse h⁻¹) was similar to the LH peak frequency measured in the control ewes, the lack of differences in LH secretion between control ewes and ewes treated with pulsatile GnRH was not surprising. These findings emphasize the importance of pulsatile GnRH administration in sheep to maintain gonadotrophin release. In contrast, LH secretory patterns in ewes treated with constant GnRH suggest GnRH receptor downregulation on the basis of the significant increase in LH peak width and amplitude on day 1, which subsided by the end of day 1 and did not recur through to day 5.

LH secretory patterns in mares did not reflect changes characteristic of GnRH receptor downregulation or desensitization during any period throughout both experiments. However, data from ewes treated with continuous GnRH in Expts 1 and 2 indicated a decrease in LH release during continuous GnRH administration that may be due to GnRH receptor desensitization or downregulation. These findings reaffirm those from a previous study that concluded that GnRH receptor downregulation occurred after exposure to high concentrations of continuous GnRH in ewes (Nett *et al.*, 1981). GnRH receptor downregulation and a decrease in amount of LH released has been described in a variety of species during continuous stimulation with exogenous GnRH. In ewes infused continuously with GnRH (2.5 μ g h⁻¹) for 12 or 24 h, GnRH receptor downregulation was observed as LH concentrations initially rose in response to GnRH infusion and then returned

to baseline within 5 h of the onset of GnRH infusion (Nett *et al.*, 1981). Similar results have been observed in ovariectomized ewes (Rippel *et al.*, 1974), pro-oestrous rats (Garner and Blake, 1979) and male rats (Clayton *et al.*, 1985). These studies emphasize the importance of pulsatile GnRH secretion for maintenance of gonadotroph secretion (Knobil, 1980; Levine and Ramirez, 1980; Moenter *et al.*, 1992). The effect of continuous GnRH infusion on GnRH receptor downregulation in horses has not been shown. In one study, over 75% of anoestrous mares injected subcutaneously with slow release implants designed to release 30 or 60 µg of a potent GnRH analogue for 28 days ovulated within 3–18 days of the start of treatment (Allen *et al.*, 1987). Similar results have been described in other studies using slow release implants containing GnRH (Ainsworth and Hyland 1991; Turner and Irvine 1991). These studies have involved the use of potent GnRH agonists and analogues, and their results have indicated decreased sensitivity to receptor downregulation by gonadotrophs. However, differential binding by potent GnRH analogues and agonists to GnRH receptors may prevent normal physiological responses by gonadotrophs (Janovick *et al.*, 1993). In addition, no studies have described the gonadotrophic response to natural GnRH administration (pulsatile or continuous) in pony mares. A clear understanding of the response of the pituitary to natural GnRH is necessary for the appropriate use of GnRH in equine reproduction.

In conclusion, mares appeared to be significantly less sensitive than ewes to extinction of LH secretion during continuous GnRH challenge. Pulsatile administration of GnRH (25 µg h⁻¹) and continuous infusion of GnRH (100 µg ml⁻¹) elicited similar LH responses in progesterone-treated, ovariectomized pony mares. These findings, together with the irregularly episodic release of LH in pony mares suggest differences between pony mares and sheep in their gonadotrophic response to administration of natural GnRH. These differences may involve structural differences in the GnRH receptor, distinct second messenger systems or variations in receptor synthesis and recycling. Future work is needed to evaluate structural differences between the ovine and equine GnRH receptor and receptor studies are needed to evaluate receptor kinetics directly.

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