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Effect of Suppression of FSH with a GnRH Antagonist (Acyline) Before and During Follicle Deviation in the Mare

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Contents

A GnRH antagonist (Acyline) was used to study the role of FSH in early development of a follicular wave in 61 mares. In Experiment 1, a single dose of 3 mg per mare, compared with 0 and 1 mg, suppressed both the FSH and follicle responses to exogenous GnRH. In Experiment 2, high concentrations of FSH were induced by two successive ablations of all follicles ≥ 6 mm on days 10 and 13 (day 0 = ovulation). A single treatment with Acyline resulted in significantly greater suppression of plasma concentrations of FSH than a single treatment with charcoal-extracted follicular fluid (source of inhibin) or oestradiol. Suppression of FSH was not significantly different between the group treated with Acyline alone and a group treated with a combination of Acyline, inhibin and oestradiol. In Experiment 3, all follicles were ablated on day 10 to induce an FSH surge and a new follicular wave. Acyline treatment on day 10 resulted in an immediate decrease in FSH, without a significant effect on day of emergence of a new wave or growth of follicles from 7 to 11 mm on days 11–13. Treatment on day 15, a day before expected follicle deviation and after the peak of the wave-stimulating FSH surge, resulted in an immediate decrease in FSH and cessation of follicle growth. Results indicated that growth of follicles for about 2 days after wave emergence was independent of FSH. In contrast, during the decline in the wave-stimulating FSH surge and before follicle deviation, growth of follicles was dependent on FSH.

Introduction

The ovulatory follicular wave in mares begins to develop at the middle of an interovulatory interval of 22–24 days (Ginther et al. 2003). After emergence of the wave, the follicles grow in a common-growth phase for several days culminating in a change in growth rates known as deviation. Deviation is characterized by continued growth of the developing dominant follicle and reduced growth and atresia of remaining (subordinate) follicles and begins when the future dominant and largest subordinate follicles are at an average diameter of 22.5 and 19.0 mm, respectively.

The main pituitary hormone involved in the stimulation and regulation of follicle development is FSH in mammals (Padmanabhan and McNeilly 2001), including horses (Ginther et al. 2003). An FSH surge is associated with the emergence of a follicular wave in mares. The concentrations decrease for 3 or 4 days before the beginning of deviation and reach a nadir 2 or 3 days later. When all follicles ≥ 6.0 mm are ablated 10 days after ovulation, FSH increases within 2 days and is associated with emergence of a new follicular wave (Gastal et al. 1997; Bergfelt et al. 2001).

The secretion of gonadotropins (FSH and LH) from the pituitary is regulated by a hypothalamic decapeptide, GnRH (Schally et al. 1971). The GnRH antagonists are synthetic peptide analogues that competitively inhibit GnRH action at pituitary receptor sites (Heber et al. 1982; Herbst et al. 2002) and thereby suppress the secretion of gonadotropins. The GnRH antagonist Acyline is a long-acting member of the azaline B family (Rivier et al. 1995). Acyline has been used to interfere with the effects of GnRH in rodents (Jiang et al. 2001), monkeys (Ramaswamy et al. 2003), humans (Herbst et al. 2002), cattle (Gumen et al. 2004) and dogs (Valiente et al. 2007). To our knowledge, Acyline has not been used in mares, although several other GnRH antagonists have been used in mares to depress plasma FSH (Palmer and Quellier 1988; Watson et al. 2000; Evans et al. 2002a,b; Guillaume et al. 2002; Briant et al. 2003).

In addition to hypothalamic regulators, ovarian hormones influence the pituitary release of FSH (Padmanabhan and McNeilly 2001). In mares, comparisons among control, follicle-ablation, and ovariectomy groups (Ginther et al. 2005) and administration of oestradiol (Ginther et al. 2007) and progesterone (Gastal et al. 1999a, 2000) were interpretable on the basis of a negative effect of oestradiol and progesterone on systemic gonadotropin concentrations throughout the oestrous cycle. The negative effect on FSH was attributable to inhibin with a contribution of oestradiol beginning near the time of deviation. The administration of a proteinaceous fraction of follicular fluid as a source of inhibin suppresses circulating concentrations of FSH in mares by 6 h after the treatment (Miller et al. 1979; Bergfelt and Ginther 1985), and exogenous oestradiol reduces FSH within 3–8 h after treatment (Miller et al. 1981; Donadeu and Ginther 2003).

The objectives of the present studies were: (i) to determine an Acyline dose that after one treatment effectively reduces FSH concentrations and affects follicle dynamics (Experiment 1), (ii) to compare the effects of Acyline, inhibin and oestradiol on inducing suppression of plasma FSH (Experiment 2) and (iii) to determine the effect of Acyline-reduced FSH concentrations on follicular development from emergence at 6 mm to a few days after the beginning of follicle deviation (Experiment 3).

Materials and Methods

Animals, ultrasonography and hormones

The mares were non-lactating mixed breeds of large ponies and apparent pony-horse crosses in good body

condition, aged 9–17 years and weighing 250–400 kg. Mares with docile temperament and no apparent abnormalities of the reproductive tract, as determined by ultrasound examinations (Ginther 1995), were used. The mares were kept under natural light in an open shelter and outdoor paddock and were maintained on alfalfa/grass hay with free access to water and trace-mineralized salt. Experimental procedures were performed in accordance with the Guide for Care and Use of Agricultural Animals in Agricultural Research and Teaching.

A real-time B-mode ultrasound scanner (Aloka SSD-900; Aloka America, Wallingford, CT, USA) with a linear-array 5.0-MHz transducer was used for transrectal examination of ovaries as described by Ginther (1995). The day of ovulation was determined by disappearance of the dominant follicle with subsequent development of a corpus luteum. Diameter of the follicles was measured with the electronic calipers at the apparent maximal area on the two-dimensional image, using the average of height and width from two frozen images. For Experiments 2 and 3, a new follicular wave was induced by transvaginal ultrasound-guided aspiration of contents of all follicles ≥ 6 mm on day 10 (ovulation = day 0) in mares as described by Gastal et al. (1997).

The GnRH antagonist, Acyline (CBD 3883H, NICHHD/NIH), was dissolved in 5% aqueous solution of D-mannitol (M-9546; Sigma Chemical Co., St Louis, MO, USA) and given as a single intramuscular injection of 3 ml. Oestradiol-17β was prepared by dissolving 50 mg in 10 ml of benzyl alcohol and adding 40 ml of safflower oil, resulting in a stock solution of 1 mg/ml, as described by Ginther et al. (2000). Charcoal-extracted bovine follicular fluid was prepared as reported previously (Welschen et al. 1977; Miller et al. 1979). Briefly, follicular fluid was obtained by aspiration of follicle contents from slaughterhouse ovaries, and steroid extraction was done by adding charcoal (50 mg/ml follicular fluid), stirring for 1 h at room temperature, centrifuging at $12000 \times g$ for 45 min at 4°C, and filtering. Charcoal extraction has been reported to remove more than 99% of steroids from follicular fluid (Welschen et al. 1977; Miller et al. 1981).

Experiment 1

This experiment was done to determine a dose of Acyline that reduced systemic concentrations of FSH and negatively affected follicle development. Mares were in the transitional reproductive phase preceding the ovulatory season as determined by the month of the year (beginning of March in the northern hemisphere), size of the largest follicle (< 30 mm), absence of a detectable corpus luteum and no record of ovulation during the previous month. Mares (n = 4 per group) were randomized into three Acyline groups. The Acyline groups received 0 mg per mare (vehicle only, 0-mg group), 1 mg per mare (1-mg group) or 3 mg per mare (3-mg group) in 3 ml of D-mannitol vehicle. At hour 0, each mare was given a single treatment of 100 μg of GnRH on each of four consecutive days (1st, 2nd, 3rd and 4th days). Mares were treated intramuscularly with Acyline or vehicle at hour-2 of the 1st day, only. Blood samples from each mare were collected from a jugular vein at hour-2 (just before Acyline treatment on the 1st day) and at hours 0, 1, 2, 3, 4, 5 and 6, relative to the injection of GnRH on each of the 4 days. Diameter of the largest follicle per mare was recorded on each of the 4 days at the hour of the GnRH treatment (hour 0).

Experiment 2

This experiment was done in preparation for Experiment 3 to determine the efficacy of Acyline on suppressing the systemic concentrations of FSH when compared with an inhibin source (charcoal-extracted follicular fluid) and oestradiol. Cyclic mares were randomized into a control group (n = 5 mares) and four treatment groups (n = 4 per group). Follicles ≥ 6 mm were ablated in each mare on day 10 and again on day 13 to produce elevated plasma FSH concentrations (Ginther et al. 2005). At hour 0 on day 14, treatment groups received either a single treatment of charcoal-extracted follicular fluid (15 ml per mare, i.v; inhibin group), oestradiol (1 mg per mare, i.m; E2 group), Acyline (3 mg per mare, i.m; Acyline group) or a combination of charcoal-extracted follicular fluid, oestradiol and Acyline (ALL group). The doses of charcoal-extracted follicular fluid (Miller et al. 1979) and oestradiol (Miller et al. 1981) were based on previous reports. Control mares received follicle ablation on days 10 and 13 but were not treated on day 14. Blood samples were collected daily from days 10 to 18. Frequent blood samples were also collected on day 14 at hours 0 (just before treatment), 1, 2, 4, 6, 8, 10 and 12. Diameter of the largest follicle was recorded daily from days 10 to 18.

Experiment 3

This experiment was done to reduce FSH concentrations with Acyline on two different days during a follicular wave to study the role of FSH in follicle development. In each mare, follicles ≥ 6 mm were ablated on day 10 to initiate a new ovulatory wave. Mares were randomized into three groups (controls, n = 14; Acyline treatment on day 10, n = 7; Acyline treatment on day 15, n = 7). Acyline (3 mg per mare, i.m.) was given either on the day of follicle ablation (day-10 group) or 5 days after ablation (day-15 group). Days 10 and 15 were selected to study the follicle effect of FSH reduction just before the beginning of wave emergence at 6 mm and just before the expected beginning of deviation on day 16 (Gastal et al. 1997). Controls received follicle ablations but no Acyline or vehicle. Jugular blood samples were collected daily on days 10 (day of ablation) to 18, and transrectal ultrasound examinations were performed every day from day 10 to the day of ovulation. Data were statistically analysed separately for days 10–15 and days 15–18.

Hormone assays

Blood samples were centrifuged ($1500 \times g$ for 10 min), and the plasma was recovered and stored (−20°C) until

assay. Plasma samples were assayed for FSH by radioimmunoassay as validated (Freedman et al. 1979) and modified (Donadeu and Ginther 2002) in our laboratory. Concentrations of immunoreactive (ir) inhibin in plasma were measured by a double antibody radioimmunoassay kit (Institute of Reproduction and Development, Monash Medical Centre, Clayton, Vic., Australia) as described by Donadeu and Ginther (2001). Intra- and interassay coefficients of variation (CV) and sensitivity for FSH were 6.5%, 9.7% and 1.1 ng/ml for Experiment 1; 7.4%, 5.1% and 4.4 ng/ml for Experiment 2 and 7.6%, 3.9% and 1.2 ng/ml for Experiment 3, respectively. Intra- and interassay CV and sensitivity for ir-inhibin in Experiment 3 were 5.3%, 6.3% and 6.8 ng/ml, respectively.

Statistical analyses

Normal distribution of the data was achieved by logarithmic transformation when indicated. Extreme observations were tested for outliers by Dixon's test (Dixon and Massey 1983). Sequential data were analysed by the SAS MIXED procedure (version 9.1.3; SAS Institute Inc., Cary, NC, USA) to determine the main effects of group and time (hour or day), and their interaction. A combination of autoregressive covariance structure within animals and a random effect between animals was modelled. A significant main effect allowed further testing and comparison of least square means using the Tukey–Kramer test inside the model. When an interaction was obtained, Student's unpaired and paired *t*-tests were used to compare two means between and within groups, respectively, and Duncan's multiple range test was used to compare more than two treatment means at a time point. A probability of $p \leq 0.05$ indicated that a difference was significant and a probability between $p > 0.05$ and $p \leq 0.1$ indicated that significance was approached. Data are presented as the mean ± SEM, unless otherwise specified.

Results

Experiment 1

Concentrations of FSH analysed over the 4 days indicated a main effect of day ($p < 0.0001$) and a group-by-day interaction ($p < 0.0001$; Fig. 1). Concentration at hour −2 (hour of Acyline treatment) on the 1st day was not different from concentrations at hour 0 on the 2nd to 4th days in the 0-mg and 1-mg groups. However, the concentrations were greater ($p < 0.05$) in 3-mg group at hour 0 on the 3rd and 4th days. There was a progressive decrease over days in the FSH response to GnRH treatment in the 0-mg group, a progressive increase in FSH in the 3-mg group, and an intermediate response in the 1-mg group. Maximal concentrations occurred 1 h after GnRH administration (hour 1 each day) and progressively decreased in the 0-mg group on each day. Concentra-

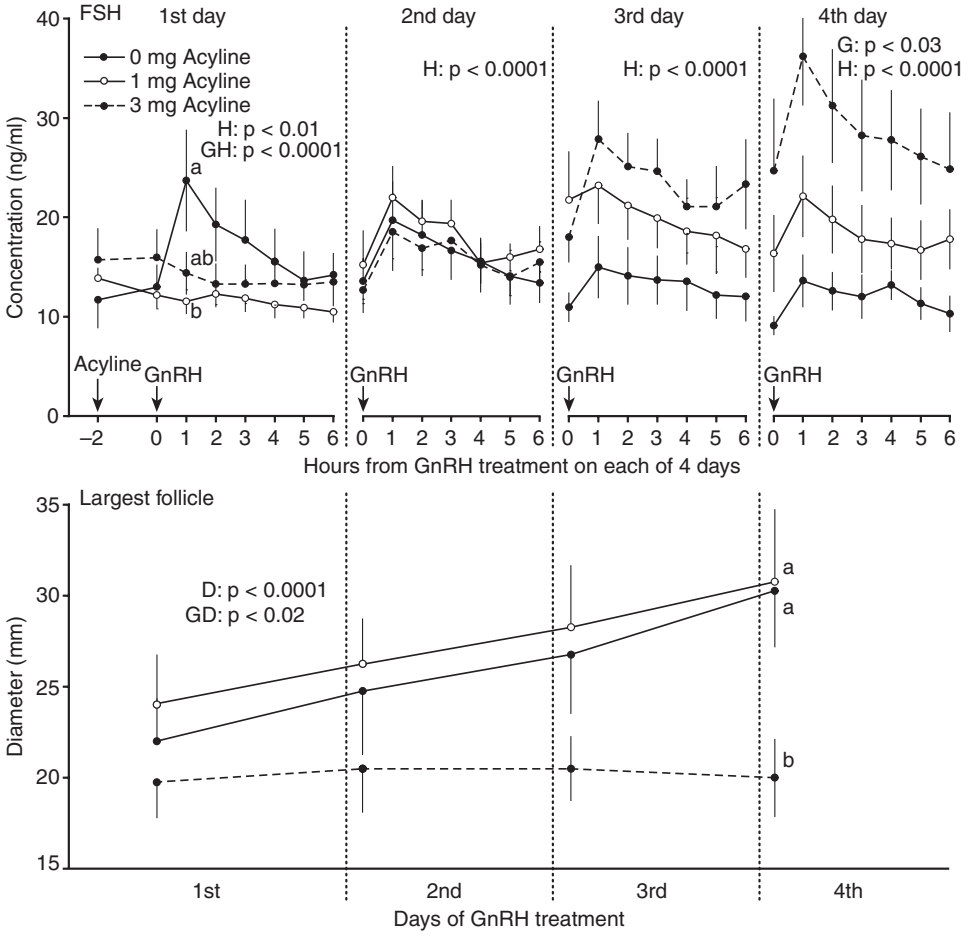

Fig. 1. Mean (±SEM) circulating concentrations of FSH and diameter of the largest follicle in mares treated with a single injection of 0, 1 or 3 mg Acyline at hour −2 on the 1st day (n = 4 mares per group). A GnRH injection was given at hour 0 on each day. The significant main effects of group (G) and hour (H) or day (D) and the interactions (GH and GD) are shown. Different letters (a, b) indicate a difference ($P < 0.05$) among groups when an interaction was obtained (Experiment 1)

tion at hour 1 in the 1-mg group was greater ($p < 0.05$) on the 2nd through 4th days than on the 1st day, but the 2nd through 4th days were not different from each other. In the 3-mg group, concentrations at hour 1 on the 1st and 2nd days were similar but increased ($p < 0.05$) on the 3rd and 4th days.

Changes in hourly concentrations of FSH in response to GnRH were analysed separately for each day, and significant main effects and interactions are shown in Fig. 1. For the 1st day, the interaction reflected an increase ($p < 0.05$) in FSH concentrations after GnRH treatment in the 0-mg Acyline-treated group from hours 0 to 1 and no significant changes in the 1-mg and 3-mg Acyline-treated groups. The FSH concentrations in the 0-mg group decreased ($p < 0.05$) to baseline values by hour 4. For the 2nd day, the main effect of hour was from an increase ($p < 0.05$) in FSH between hours 0 and 1 in each of the three groups after GnRH treatment. The sources of the main effect of hour for the 3rd day and the main effects of group and hour for the 4th day are apparent (Fig. 1).

Diameter of the largest follicle did not differ among treatment groups at hour 0 of the 1st day (combined, 21.9 ± 1.3 mm). Diameter on each of the 4 days and the statistical results are presented in Fig. 1. The interaction of group by day reflected a progressive increase in diameter in 0-mg and 1-mg groups and no increase in the 3-mg group. The diameter of the largest follicle on the 4th day was greater ($p < 0.05$) in 0-mg group (30.2 ± 3.0 mm) and 1-mg group (30.7 ± 3.9 mm) than in 3-mg group (20.0 ± 2.1 mm). There were no apparent adverse reactions at the Acyline injection site.

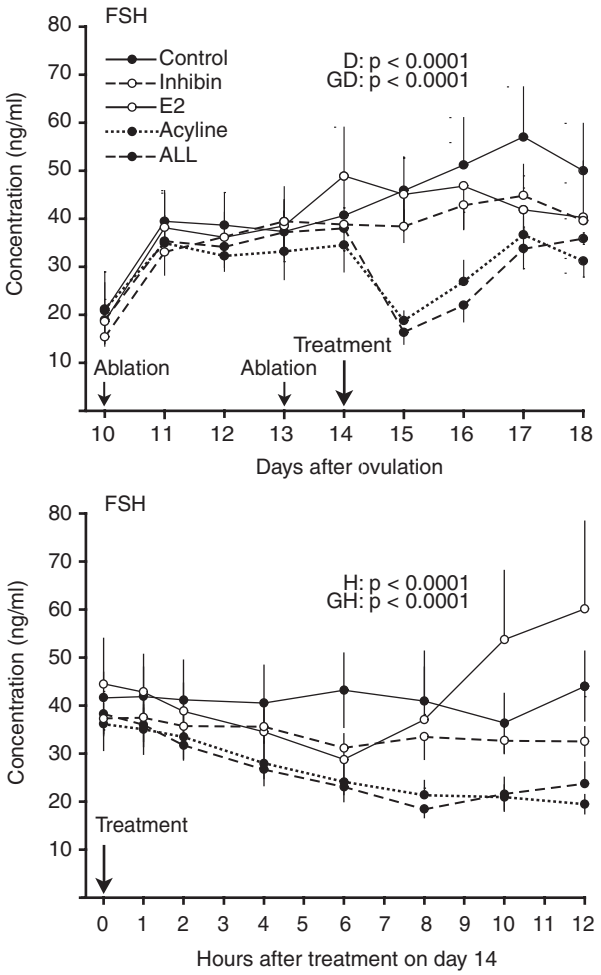

Fig. 2. Mean (±SEM) circulating concentrations of FSH daily for days 10–18, and hourly for hours 0–12 on day 14 in controls (n = 5 mares) and in mares treated with charcoal-extracted follicular fluid (15 ml per mare; inhibin group), oestradiol (1 mg per mare; E2 group), Acyline (3 mg per mare; Acyline group) or a combination of charcoal-extracted follicular fluid, oestradiol and Acyline (ALL group) on day 14 (n = 4 mares per group). The significant main effects of group (G) and hour (H) or day (D) and the interactions (GH and GD) are shown (Experiment 2)

Experiment 2

There were no significant differences in diameter of the largest follicle among different treatment groups from days 14 to 18 (data not shown). The FSH concentrations for days 10–18 and the probabilities for significant main effects and an interaction are shown in Fig. 2. The group-by-day interaction was significant reflecting an increase ($p < 0.05$) between days 10 and 11 and a plateau on days 11–14 in each group, an increase ($p < 0.05$) between days 14 and 17 in the control group, no change after day 14 in the inhibin and E2 groups, and a decrease ($p < 0.05$) on day 15 and then an increase ($p < 0.05$) in the Acyline and ALL groups. On day 15, the concentration was lower ($p < 0.05$) in the Acyline and ALL groups than in the inhibin, E2 and control groups. On days 17 and 18, there were no differences in FSH concentrations among groups.

The hourly changes in the concentrations of FSH for hours 0–12 on the day of treatment (day 14) also showed a significant interaction (Fig. 2). The interaction resulted from constant concentrations in controls; a decrease ($p < 0.05$) after hour 0 in the inhibin, E2, Acyline and ALL groups; and an apparent increase ($p < 0.06$) between hours 6 and 12 in the E2 group. Concentrations of FSH at hour 12 were lower ($p < 0.05$) in the Acyline and ALL groups than in the controls.

Experiment 3

The Acyline-treated day-15 group was included as part of the control group until the day of treatment. As a result, the number of mares in the control group was 21 mares from the beginning of the experiment until day 15 and 14 mares from days 15 to 18. Data for FSH, follicle diameter and ir-inhibin for days 10–15 and separately for days 15–18 and the probabilities for main effects and interactions are shown in Fig. 3.

The FSH concentrations on days 10–15 showed an interaction because of a progressive increase ($p < 0.01$) from days 10 to 14 in the control group vs a decrease ($p < 0.02$) on day 11, followed by an increase ($p < 0.05$) to control concentrations by day 14 in the Acyline-treated day-10 group. For days 15–18, an interaction was due to a progressive decrease in FSH that began on day 14 and continued to day 18 in the controls and the Acyline-treated day-10 group vs a decrease ($p < 0.01$) between days 15 and 16 and then an

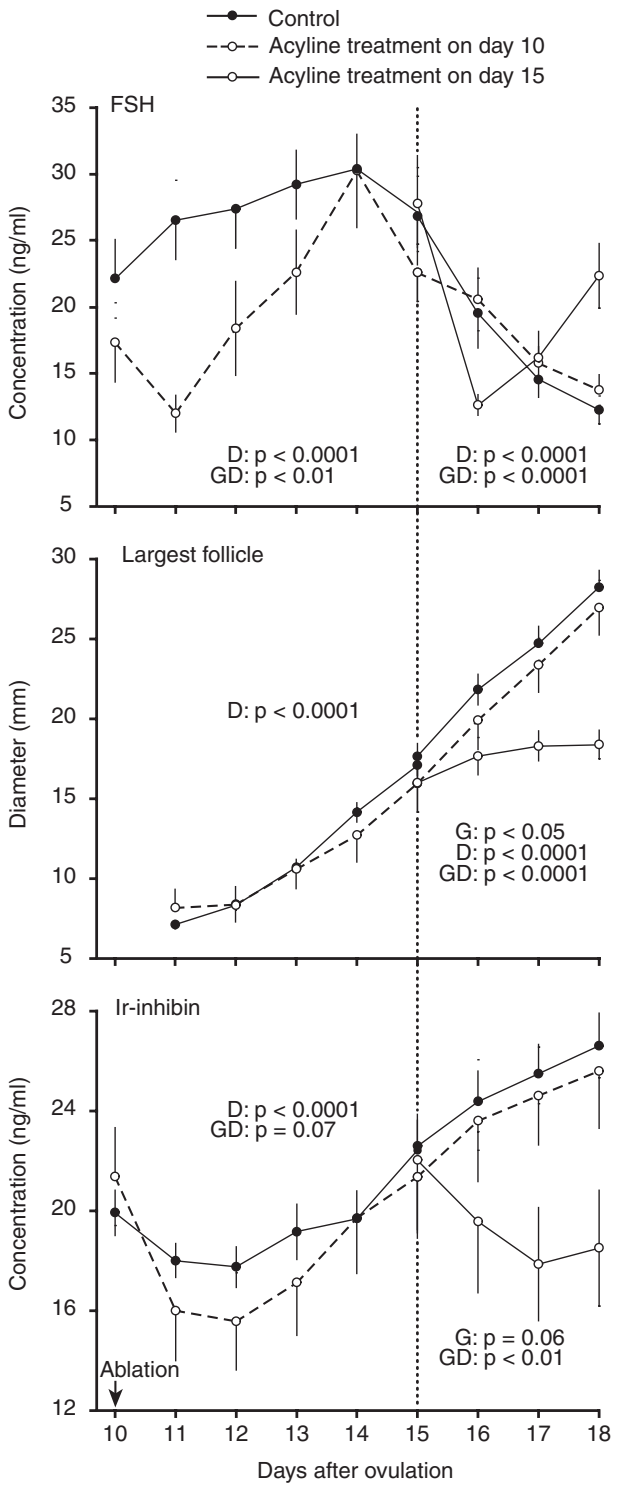

Fig. 3. Mean (±SEM) circulating FSH concentrations, follicle diameter and ir-inhibin concentrations in controls (n = 21 mares on days 10–15 and n = 14 mares on days 15–18) and in mares with a single treatment of 3 mg Acyline on day 10 (n = 7) and on day 15 (n = 7). The significant main effects of group (G) and day (D) and the interaction (GD) are shown (Experiment 3)

increase ($p < 0.04$) in the Acyline-treated day-15 group. On day 18, concentration was higher ($p < 0.01$) for the day-15 group than for both the controls and the day-10 group.

Diameter of the largest follicle increased (main effect of day) similarly in the control and Acyline-treated day-10 groups between days 10 and 15 (Fig. 3). For days 15–18, the interaction was due to a continued increase ($p < 0.0001$) in the diameter of the largest follicle in the controls and the Acyline-treated day-10 group and no significant change in the Acyline-treated day-15 group after day 16. Diameter of the follicle did not differ among groups on day 15 (overall, 16.8 ± 0.6 mm) but was smaller ($p < 0.05$) on day 18 in the Acyline-treated day-15 group (18.4 ± 0.9 mm) than in the controls (28.2 ± 1.0 mm) and Acyline-treated day-10 group (26.9 ± 1.7 mm). Follicle data were not available for one mare after day 18. The follicle regressed after day 18 in five of six remaining mares in the Acyline-treated day-15 group, and ovulation occurred from a subsequent follicular wave. The controls (13.9 ± 0.4 days) and the Acyline-treated day-10 group (14.1 ± 0.3 days) had shorter ($p < 0.05$) intervals from ablation to ovulation than the Acyline-treated day-15 group (18.8 ± 1.1 days). The interval from Acyline treatment to ovulation (overall 14.0 ± 0.5 days) and the growth rate of the largest follicle for the 7 days before ovulation (overall 2.7 ± 0.3 mm/day) were similar for the two Acyline-treated groups.

For ir-inhibin concentrations on days 10–15, the main effect of day was from an overall decrease ($p < 0.01$) combined for the control and Acyline group by day 12, followed by an increase ($p < 0.01$; Fig. 3). The interaction approached significance owing to an apparent decrease to a lower concentration in the Acyline group. For days 15–18, an interaction reflected a progressive increase ($p < 0.05$) in ir-inhibin concentrations in the controls and Acyline-treated day-10 group vs a decrease ($p < 0.01$) on days 15–17 in the Acyline-treated day-15 group. Concentration of ir-inhibin on day 18 was lower ($p < 0.05$) in the Acyline-treated day-15 group than in the other groups.

Discussion

The GnRH antagonist (Acyline) has not been used previously in horses, and therefore an appropriate dose was determined that would suppress both a GnRH-induced increase in FSH and follicle growth. Several studies have described the ability of GnRH to stimulate FSH secretion and follicle development in mares (Evans and Irvine 1977; Palmer and Quellier 1988; Ginther and Bergfelt 1990). The GnRH challenge was given on four consecutive days with administration of Acyline only on the 1st day to assess the duration of the Acyline FSH inhibitory effect. Both the 1-mg and 3-mg doses of Acyline were effective in suppressing the GnRH-induced FSH increase 2 h after administration of the antagonist but only on the 1st day. The increase in diameter of the largest follicle to >30 mm by the 4th day in mares treated with 0 or 1 mg Acyline is attributable to the FSH stimulatory effect of repeated GnRH treatment. In the mares treated with 3 mg, the largest follicle (not necessarily the same follicle) did not increase in diameter and plateaued at approximately 20 mm, despite the enhanced GnRH stimulation of FSH on the 3rd and 4th days. In the 0-mg group, the secretion of the

FSH-suppressing factors inhibin and oestradiol by the growing follicles (Ginther 1992; Bergfelt and Ginther 1993; Donadeu and Ginther 2003; Ginther et al. 2005) likely accounts for the progressing diminished FSH response to GnRH over days. In contrast, in mares treated with 3 mg Acyline, the concentrations of FSH were greater than in the 0-mg group by the 4th day. Our favoured interpretation is that the follicles in this group became defective, as indicated by the lack of a diameter increase in the largest follicle, and therefore did not produce an adequate quantity of FSH suppressors. Presumably, the FSH suppression on the 1st day from 3 mg Acyline, compared with 1 mg, was more prolonged, resulting in a detrimental effect on the follicles. The intermediate FSH values for the 1-mg group could be attributed to individual variability in the response to the low Acyline dose. Results from Experiment 1 indicated that Acyline would be a useful research tool to study the hypothalamus-hypophysis-gonad axis in the mare. The 3-mg dose was chosen for use in Experiments 2 and 3, owing to the inhibitory effects on both FSH concentrations and follicle growth.

In Experiment 2, the increase in FSH concentrations in the controls following follicle ablation on day 10 and again on day 13 is in accordance with previous reports (Gastal et al. 1997; Ginther et al. 2005). The FSH surge was less pronounced after ablation on day 13 than on day 10, presumably because of the higher FSH concentrations at day 13. The FSH increases in the follicle-ablated controls are attributable to the removal of FSH-suppressing factors of follicle origin. Inhibin, rather than oestradiol, has been shown to be the FSH inhibitor during the early development of a follicular wave (Ginther et al. 2003, 2005). Although oestradiol was not assayed in the present experiment, it does not begin to increase in the circulation until 1 or 2 days before deviation (Gastal et al. 1999b; Bergfelt et al. 2001). Deviation was not expected to begin until 6 days after the day-13 ablation (Gastal et al. 1997) or near the end of the experiment on day 18.

On day 15 in both the Acyline group and the ALL group, FSH concentrations were suppressed approximately 50% of the pretreatment values. Similar suppression in FSH was reported for ovariectomized mares immunized against GnRH (Garza et al. 1986) and in ovariectomized mares treated with the GnRH antagonist, Antarelix (Watson et al. 2000). The FSH increase after day 16 indicated that the suppression was no longer effective by 2 days after treatment. Both inhibin and oestradiol adequately suppressed FSH to prevent the post-ablation increase in FSH that occurred in the controls. These results are consistent with the suggested FSH suppressor activities of these factors during the ovulatory follicular wave (Ginther et al. 2004). However, the daily sampling indicated that the GnRH antagonist, Acyline, was a more effective FSH suppressor than either inhibin or oestradiol. Acyline alone reduced the FSH concentration to the day-10 pre-ablation concentration; adding inhibin source and oestradiol to Acyline did not further depress FSH. Therefore, Acyline alone was used as the FSH suppressor in Experiment 3.

When the day-14 post-ablation concentrations were determined every 1 or 2 h, charcoal-extracted follicular fluid suppressed circulating FSH by 16% at hour 6. The suppressive activity of the charcoal-extracted follicular fluid is attributable to its inhibin content (Miller et al. 1979; Bergfelt and Ginther 1985). Inhibin has a known negative feedback effect on FSH in mares (Ginther et al. 2003), and peripheral concentration of immunoreactive inhibin and FSH have an approximate inverse relationship during the equine oestrous cycle (Bergfelt et al. 1991; Roser et al. 1994; Nagamine et al. 1998). In a previous study (Miller et al. 1979), similar results were reported with a suppression of 14% in FSH concentrations by hour 6 after treatment with 20-ml charcoal-extracted follicular fluid to ovariectomized mares. Similar to inhibin, oestradiol induced a suppression of FSH concentrations and the values were reduced by 35% at hour 6 after treatment. This is consistent with a previous study (Miller et al. 1981), where the same oestradiol dose decreased FSH by 25% by 8 h post-treatment in ovariectomized mares. The present results from the frequent sampling during hours 0–12 indicated that oestradiol reduced the FSH concentrations below initial concentrations parallel with the effect of Acyline, but the suppression was of shorter duration. In a reported study, the FSH depression from the same dose of oestradiol was followed by a rebound in FSH, reaching concentrations higher than pretreatment concentrations by 12 h (Miller et al. 1981). In the present study, there was an increase in FSH by hour 12 in the E2 group, but significance was only approached ($p = 0.09$). The mares in the Acyline and ALL groups showed a continuous FSH decrease to approximately 50% of the pretreatment values.

In Experiment 3, the similarity between the Acyline-treated and control mares in post-ablation growth rate of the largest follicle between days 11 (diameter, 7.3 ± 0.3 mm) and 13 (10.7 ± 0.5 mm), despite the FSH suppression in the Acyline group, indicated that the follicles were relatively FSH independent at this stage. This result is consistent with the results of studies utilizing post-ablation changes in follicle growth and FSH concentrations (Donadeu and Ginther 2003), GnRH immunization (Imboden et al. 2006), and repeated administration of the GnRH agonist goserelin (Pedersen et al. 2002). Similarly, the ir-inhibin profile was not affected by Acyline treatment at this time and is attributable to the small follicles at the beginning of the follicular wave. In this regard, previous studies have reported that inhibin concentrations increase with follicle growth (Goudet et al. 1999), but do not reach circulating concentrations that are sufficient to initiate a decline in the wave-stimulating FSH surge until the largest follicle reaches 12 or 13 mm (Bergfelt et al. 2001; Donadeu and Ginther 2003). Similarly, in the present experiment the peak of the FSH surge in the control and Acyline-treated day-10 groups occurred on day 14, when the diameter of the largest follicle was 13.8 ± 0.6 mm. By that time, ir-inhibin was at pre-ablation concentrations and increased rapidly thereafter in temporal association, with the decrease in FSH.

In contrast to the relative FSH independence before 12 mm, the follicle was dependent on GnRH-dependent FSH after day 15 (largest follicle, 16.8 ± 0.6 mm). This was indicated by the FSH suppression and cessation of

follicle growth between days 15 and 18 in the Acyline-treated day-15 group. The decrease in ir-inhibin in the day-15 group was attributable to the cessation of follicle growth. Regression of the follicles in the day-15 group indicated that GnRH-dependent FSH was needed to maintain follicular viability even after the FSH peak; that is, the declining FSH concentrations were required for continued follicle growth. The only follicle that continued growing was in a mare that had the smallest follicle on day 15 (13.2 mm), consistent with FSH independence shown in the day-10 group. The demise of the largest follicle in the day-15 group was attributed to inadequate FSH. Inadequate LH is not likely to have been involved, based on reports (Gastal et al. 1999a; Bergfelt et al. 2001) that under experimentally reduced LH concentrations, the follicles grew at the same rate as in controls until the day of deviation but not thereafter. The increase in FSH after day 16 in the group treated with Acyline on day 15, presumably represented the beginning of a new FSH surge in association with the emergence of a new follicular wave. The emergence of a new wave accounts for the approximately 5-day longer interval from ablation at day 10 to ovulation.

In conclusion, a single 3-mg dose of Acyline suppressed the FSH-inducing and follicle-stimulating effects of exogenous GnRH. Treatment with the 3-mg dose during the high FSH concentrations after follicle ablation resulted in a more profound and longer decrease in FSH than a single treatment with charcoal-extracted follicular fluid (source of inhibin) or oestradiol. After ablating all follicles ≥ 6 mm on day 10 post-ovulation, Acyline induced an immediate decrease in FSH, without delaying the emergence of a new follicular wave. The FSH decrease did not deter wave emergence and growth of the largest follicle from 6 to 11 mm, indicating that early development of the follicular wave was GnRH-dependent FSH independent. However, treatment on day 15 (after the peak of the FSH surge and before expected deviation) resulted in an immediate decrease in FSH and retardation of follicle growth, indicating that the follicles had become dependent on GnRH-dependent FSH.

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Author contributions

All authors contributed.

References

Bergfelt DR, Ginther OJ, 1985: Delayed follicular development and ovulation following inhibition of FSH with equine follicular fluid in the mare. Theriogenology **24,** 99–108.

Bergfelt DR, Ginther OJ, 1993: Relationships between FSH surges and follicular waves during the estrous cycle in mares. Theriogenology **39,** 781–796.

Bergfelt DR, Mann BG, Schwartz NB, Ginther OJ, 1991: Circulating concentrations of immunoreactive inhibin and FSH during the estrous cycle of mares. J Equine Vet Sci **11,** 319–322.

Bergfelt DR, Gastal EL, Ginther OJ, 2001: Response of estradiol and inhibin to experimentally reduced luteinizing hormone during follicle deviation in mares. Biol Reprod **65,** 426–432.

Briant C, Ottogalli M, Morel M, Guillaume D, 2003: Use of a GnRH antagonist, antarelix, associated or not with hCG, to control ovulation in cyclic pony mares. Domest Anim Endocrinol **24,** 305–322.

Dixon WJ, Massey FJ, 1983: Introduction to Statistical Analysis, 4th edn. McGraw-Hill, New York.

Donadeu FX, Ginther OJ, 2001: Effect of number and diameter of follicles on plasma concentrations of inhibin and FSH in mares. Reproduction **121,** 897–903.

Donadeu FX, Ginther OJ, 2002: Changes in concentrations of follicular fluid factors during follicle selection in mares. Biol Reprod **66,** 1111–1118.

Donadeu FX, Ginther OJ, 2003: Suppression of circulating concentrations of FSH and LH by inhibin and estradiol during the initiation of follicle deviation in mares. Theriogenology **60,** 1423–1434.

Evans MJ, Irvine CH, 1977: Induction of follicular development, maturation and ovulation by gonadotropin releasing hormone administration to acyclic mares. Biol Reprod **16,** 452–462.

Evans MJ, Alexander SL, Irvine CH, Taylor TB, Kitson NE, Livesey JH, 2002a: Administration of the GnRH antagonist cetrorelix during the luteal phase of the mare. Theriogenology **58,** 527–532.

Evans MJ, Kitson NE, Alexander SL, Irvine CH, Turner JE, Perkins NR, Livesey JH, 2002b: Effectiveness of an antagonist to gonadotrophin releasing hormone on the FSH and LH response to GnRH in perifused equine pituitary cells, and in seasonally acyclic mares. Anim Reprod Sci **73,** 37–51.

Freedman LJ, Garcia MC, Ginther OJ, 1979: Influence of photoperiod and ovaries on seasonal reproductive activity in mares. Biol Reprod **20,** 567–574.

Garza F, Thompson DL, French DD, Wiest JJ, St George RL, Ashley KB, Jones LS, Mitchell PS, McNeill DR, 1986: Active immunization of intact mares against gonadotropin-releasing hormone: differential effects on secretion of luteinizing hormone and follicle-stimulating hormone. Biol Reprod **35,** 347–352.

Gastal EL, Gastal MO, Bergfelt DR, Ginther OJ, 1997: Role of diameter differences among follicles in selection of a future dominant follicle in mares. Biol Reprod **57,** 1320–1327.

Gastal EL, Bergfelt DR, Nogueira GP, Gastal MO, Ginther OJ, 1999a: Role of luteinizing hormone in follicle deviation based on manipulating progesterone concentrations in mares. Biol Reprod **61,** 1492–1498.

Gastal EL, Gastal MO, Wiltbank MC, Ginther OJ, 1999b: Follicle deviation and intrafollicular and systemic estradiol concentrations in mares. Biol Reprod **61,** 31–39.

Gastal EL, Gastal MO, Nogueira GP, Bergfelt DR, Ginther OJ, 2000: Temporal interrelationships among luteolysis, FSH and LH concentrations and follicle deviation in mares. Theriogenology **53,** 925–940.

Ginther OJ, 1992: Reproductive Biology of the Mare: Basic and Applied Aspects, 2nd edn. Equiservices Publishing, Cross Plains.

Ginther OJ, 1995: Ultrasonic Imaging and Animal Reproduction: Horses, Book 2. Equiservices Publishing, Cross Plains.

Ginther OJ, Bergfelt DR, 1990: Effect of GnRH treatment during the anovulatory season on multiple ovulation rate

and on follicular development during the ensuing pregnancy in mares. J Reprod Fertil **88,** 119–126.
Ginther OJ, Bergfelt DR, Kulick LJ, Kot K, 2000: Selection of the dominant follicle in cattle: role of estradiol. Biol Reprod **63,** 383–389.
Ginther OJ, Beg MA, Donadeu FX, Bergfelt DR, 2003: Mechanism of follicle deviation in monovular farm species. Anim Reprod Sci **78,** 239–257.
Ginther OJ, Beg MA, Gastal MO, Gastal EL, 2004: Follicle dynamics and selection in mares. Anim Reprod **1,** 45–63.
Ginther OJ, Gastal EL, Gastal MO, Beg MA, 2005: Regulation of circulating gonadotropins by the negative effects of ovarian hormones in mares. Biol Reprod **73,** 315–323.
Ginther OJ, Utt MD, Beg MA, Gastal EL, Gastal MO, 2007: Negative effect of oestradiol on luteinizing hormone throughout the ovulatory luteinizing hormone surge in mares. Biol Reprod **77,** 543–550.
Goudet G, Belin F, Bezard J, Gerard N, 1999: Intrafollicular content of luteinizing hormone receptor, alpha-inhibin, and aromatase in relation to follicular growth, estrous cycle stage, and oocyte competence for in vitro maturation in the mare. Biol Reprod **60,** 1120–1127.
Guillaume D, Bruneau B, Briant C, 2002: Comparison of the effects of two GnRH antagonists on LH and FSH secretion, follicular growth and ovulation in the mare. Reprod Nutr Dev **42,** 251–264.
Gumen A, Powell JA, Olbrich DL, Wiltbank MC, 2004: Development of ovarian follicular cysts after treatment of cattle with a GnRH receptor antagonist (Acyline) and estradiol. In: Proceedings of the 37th meeting for the Society for the study of Reproduction, Vancouver, Canada, pp. 275 Abstract 794.
Heber D, Dodson R, Swerdloff RS, Channabasavaiah K, Stewart JM, 1982: Pituitary receptor site blockade by a gonadotropin-releasing hormone antagonist in vivo: mechanism of action. Science **216,** 420–421.
Herbst KL, Anawalt BD, Amory JK, Bremner WJ, 2002: Acyline: the first study in humans of a potent, new gonadotropin-releasing hormone antagonist. J Clin Endocrinol Metab **87,** 3215–3220.
Imboden I, Janett F, Burger D, Crowe MA, Hässig M, Thun R, 2006: Influence of immunization against GnRH on reproductive cyclicity and estrous behavior in the mare. Theriogenology **66,** 1866–1875.
Jiang G, Stalewski J, Galyean R, Dykert J, Schteingart C, Broqua P, Aebi A, Aubert ML, Semple G, Robson P, Akinsanya K, Haigh R, Riviere P, Trojnar J, Junien JL, Rivier JE, 2001: GnRH antagonists: a new generation of long acting analogues incorporating p-ureido-phenylalanines at positions 5 and 6. J Med Chem **44,** 453–467.
Miller KF, Wesson JA, Ginther OJ, 1979: Changes in concentrations of circulating gonadotropins following administration of equine follicular fluid to ovariectomized mares. Biol Reprod **21,** 867–872.
Miller KF, Wesson JA, Ginther OJ, 1981: Interaction of estradiol and a non-steroidal follicular fluid substance in the regulation of gonadotropin secretion in the mare. Biol Reprod **24,** 354–358.
Nagamine N, Nambo Y, Nagata S, Nagaoka K, Tsunoda N, Taniyama H, Tanaka Y, Tohei A, Watanabe G, Taya K, 1998: Inhibin secretion in the mare: localization of inhibin alpha, beta(A), and beta(B) subunits in the ovary. Biol Reprod **59,** 1392–1398.
Padmanabhan V, McNeilly AS, 2001: Is there an FSH-releasing factor? Reproduction **121,** 21–30.
Palmer E, Quellier P, 1988: Uses of LHRH and analogues in the mare. In: 11th International Congress on Animal Reproduction and Artificial Insemination, University College Dublin, Ireland, pp. 339–344.
Pedersen HG, Berrocal B, Thomson SRM, Watson ED, 2002: Gonadotropic control of follicular growth in the mare. Theriogenology **58,** 465–468.
Ramaswamy S, Marshall GR, Pohl CR, Friedman RL, Plant TM, 2003: Inhibitory and stimulatory regulation of testicular inhibin B secretion by luteinizing hormone and follicle-stimulating hormone, respectively, in the rhesus monkey (*Macaca mulatta*). Endocrinology **144,** 1175–1185.
Rivier JE, Jiang GC, Porter J, Hoeger CA, Craig AG, Corrigan A, Vale W, Rivier CL, 1995: Gonadotropin-releasing hormone antagonists: novel members of the Azaline B family. J Med Chem **38,** 2649–2662.
Roser JF, McCue PM, Hoye E, 1994: Inhibin activity in the mare and stallion. Domest Anim Endocrinol **11,** 87–100.
Schally AV, Arimura A, Kastin AJ, Matsuo H, Baba Y, Redding TW, Nair RMG, Debeljuk L, White WF, 1971: Gonadotropin-releasing hormone: one polypeptide regulates secretion of luteinizing and follicle-stimulating hormones. Science **173,** 1036–1038.
Valiente C, Corrada Y, de la Sota PE, Gerez PG, Gobello C, 2007: Effect of the GnRH antagonist, acyline, on canine testicular characteristics. Theriogenology **68,** 687–692.
Watson ED, Pedersen HG, Thomson SRM, Fraser HM, 2000: Control of follicular development and luteal function in the mare: Effects of a GnRH antagonist. Theriogenology **54,** 599–609.
Welschen R, Hermans WP, Dullaart J, De Jong FH, 1977: Effects of an inhibin-like factor present in bovine and porcine follicular fluid on gonadotropin levels in ovariectomized rats. J Reprod Fertil **50,** 129–131.

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