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Development of One vs Multiple Ovulatory Follicles and Associated Systemic Hormone Concentrations in Mares

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Contents

Ablation of follicles ≥ 6 mm in diameter and treatment with PGF2α 10 days after ovulation were used to induce the development of ovulatory waves. Comparisons were made between induced waves with one (33 waves, 72%) and multiple (13 waves, 28%) ovulatory follicles. Diameter deviation was defined as the separation of follicles into dominant and subordinate categories. Multiple ovulatory follicles were preceded by more ($p < 0.001$) follicles ≥ 20 mm at the beginning of deviation, higher LH preceding deviation (approached significance, $p < 0.08$), lower ($p < 0.05$) concentrations of FSH on the day of deviation and thereafter, and higher ($p < 0.0003$) oestradiol by 2 days after deviation. During the peri-ovulatory period, systemic hormone concentrations for waves with multiple ovulations involved higher oestradiol before ovulation (approached significance, $p < 0.07$), lower FSH ($p < 0.04$) before and after ovulation, and both higher progesterone ($p < 0.05$) and lower LH ($p < 0.05$) beginning the day after ovulation. Results indicated that by the beginning of deviation there were more follicles ≥ 20 mm and subsequently greater oestradiol production in waves that led to the development of multiple ovulatory follicles, and during the peri-ovulatory period differences between one and multiple ovulations were consistent with the negative effects of the ovarian hormones on the gonadotropins.

Introduction

Mares are good comparative research models for follicle studies because of the many similarities they share with women in the dynamics of the ovulatory wave. The similarities include a constant relative diameter of the largest follicle at definable events throughout the follicular wave (2.1 or 2.2 times larger in mares; Ginther et al. 2004a). In both species, the follicles of the ovulatory wave undergo a common growth phase for several days that ends in the beginning of a process termed 'follicle deviation'. Deviation in mares begins when the largest follicle reaches a mean of 22.5 mm and is characterized by the continued growth of a follicle that becomes dominant and reduced growth and regression of follicles that become subordinate (Ginther et al. 2004b). Direct information on the day of emergence (e.g. at 6 mm) of the future ovulatory follicle in mares requires monitoring of individually identified follicles from examination to examination (e.g. daily). However, follicles from a previous wave overlap with the follicles of the common growth phase in about 25% of ovulatory waves (Ginther et al. 2004a). Identification of follicles is aided considerably by the ablation of follicles to induce a new wave with minimal overlapping of follicles (Gastal et al. 1997). In a recent study (Jacob et al. 2007a), the follicle and hormone data in individual mares was compared between two consecutive oestrous cycles to check for repeatability, using correlation analyses. The study of the repeatability of characteristics involving follicle emergence and the common growth phase was precluded by the overlapping of follicles from a previous wave.

In monovular species, two or more dominant follicles may develop in some follicular waves, and follicular diameters of 28 mm in horses (Ginther 1993), 13 mm in women (Ginther et al. 2004a) and 10 mm in cattle (Kulick et al. 2001) have been used as indicators of dominance. The hormonal basis and the nature of deviation associated with the development of two dominant follicles during spontaneous waves in mares were studied recently (Jacob et al. 2007b). The group with two dominant follicles had lower concentrations of FSH. A first deviation occurred between the second and third largest follicles and a second deviation occurred between the two largest follicles, an average of 2.5 days after the first deviation. The hormonal changes associated with two ovulations could not be studied because of regression of one of the two dominant follicles, beginning at the second deviation. The origin, development and hormonal aspects of multiple dominant follicles have been studied in heifers (Kulick et al. 2001; Beg et al. 2003; Acosta et al. 2005) and cows (Lopez et al. 2005). However, the first post-ovulation anovulatory wave was used in all of the studies in cattle, and therefore the hormonal changes associated with ovulation of the multiple dominant follicles were not considered.

In this study, we used ablation-induced waves for study of the follicle and systemic hormone changes associated with the development of one vs multiple ovulations. In addition, the repeatability of follicle and hormone characteristics within individual mares between consecutive postablation ovulatory waves was studied.

Materials and Methods

Animals and ultrasonography

Mares were handled according to the United States Department of Agriculture Guide for Care and Use of Agricultural Animals in Agricultural Research and Teaching. The mares were mixed breeds of large ponies and apparent pony-horses aged 5 to >17 years and weighed 250–400 kg. A total of 24 mares with a docile temperament and no apparent abnormalities of the reproductive tract, as determined by ultrasound examinations (Ginther 1995), were used in two consecutive

interovulatory intervals during July–September (Northern Hemisphere). The mares were kept under natural light in an open shelter and outdoor paddock and were maintained on alfalfa/grass hay with access to water and trace-mineralized salt. All mares remained healthy and in good body condition throughout the study.

Transrectal B-mode ultrasonographic examinations of the ovaries and measuring of follicles were done daily, by a single operator, using a real-time ultrasound scanner (Aloka SSD-900; Aloka America, Wallingford, CT, USA) with a linear-array 7.5-MHz transducer, as described (Ginther 1995). An ovulatory wave was induced 10 days after ovulation by ablation of all follicles ≥ 6 mm by transvaginal ultrasound-guided aspiration of follicle contents and stimulation of luteolysis with 5 mg of PGF2α, as described by Gastal et al. (1997, 1999a). Follicles that refilled to ≥ 15 mm on subsequent days were re-aspirated. The postablation identity of follicles ≥ 6 mm of the induced ovulatory follicular wave was maintained until the outcome of dominant follicles was known.

The beginning of the formation of a haemorrhagic anovulatory follicle (HAF) in the absence of an accompanying ovulation was considered equivalent to the day of ovulation, and both ovulation and the beginning of an HAF were designated day 0. In this regard, ultrasound indicators of impending ovulation, follicle diameter, and systemic FSH, LH, oestradiol and progesterone concentrations were similar on day −1 between a group with a single ovulation and no HAF and a group with a single HAF and no ovulation (Ginther et al. 2006a). When multiple ovulations or multiple HAFs or a combination of ovulations and HAFs occurred, the day of the first ovulation or HAF was taken to be day 0.

Characterization of waves

Retrospective examination of the diameter data profiles for individually identified follicles was used to determine the day of emergence at ≥ 6 mm of the follicle that ovulated. Follicles that reached ≥ 6 mm on the same day or consecutive days were considered part of the ovulatory wave. Follicles that skipped ≥ 1 day in an emergence sequence were not considered to be part of the wave (Gastal et al. 1999a, 2000). The day of the beginning of deviation was also determined from the diameter data profiles. The four largest follicles on the day of the beginning of deviation were designated F1, F2, F3 and F4, in descending diameter. The F1 to F4 ranking was used for analyses that involved deviation but not for ovulation. The beginning of deviation (same as end of common growth phase) was identified by the day preceding the first apparent change in the differences in diameter between follicles (Gastal et al. 1997). Days of deviation were established before hormone data were inspected or analysed. For waves with one ovulation, deviation was detected by comparing the daily diameters of F1 with the diameters of F2. For waves with two ovulations, deviation was detected by considering the diameters of both F1 and F2 vs the diameters of F3. When the beginning of deviation was readily identified, the event was defined as 'determinable deviation'. Determinable deviation was used to display the diameter growth profiles of F1 to F4 from 1 day before to 4 days after deviation within the groups with one and two ovulations. For this purpose, waves with three ovulations were omitted. When the day of deviation was undeterminable, an expected day of deviation was assigned, based on a diameter nearest to the mean diameter of F1 in 31 waves with determinable deviation (22.6 mm). The expected beginning of deviation was used when the wave contained only F1 or when F2 was undersized and was not usable as an indicator of deviation. An undersized F2 was defined by a diameter at the expected beginning of deviation that was more than two standard deviations (2 SD = ± 4.2 mm) below the F2 mean (19.9 mm) at expected deviation in waves with one ovulation.

Waves were grouped retrospectively into those with one ovulation vs multiple ovulations, considering an HAF as an ovulation. Data for gonadotropin concentrations were normalized to the beginning of expected deviation and examined from 7 days before to 3 days after deviation. Data were examined separately for 3 days before to 4 days after an ovulation (days −3 to 4). The 3 days after the beginning of deviation and 3 days before ovulation were used to represent the mean 6-day interval between deviation and day 0. For oestradiol concentrations, 12 induced waves with determinable deviation were randomly selected from the group with one ovulation and all nine waves with determinable deviation were used in the group with multiple ovulations; assays were performed from 2 days before to 2 days after deviation and on days −2, 0, 2 and 4. For progesterone concentrations, induced waves with determinable deviation were assayed for 2, 1 and 0 days before deviation and for days 0, 1, 2, 3 and 4 relative to the first ovulation.

The group with one ovulation was retrospectively subgrouped into waves with one follicle or an undersized F2 vs waves with multiple follicles at the beginning of expected deviation. Expected deviation was used to provide a common reference point for both one and multiple follicles. The group with multiple ovulations was not used so that results would reflect the number of follicles at deviation, without the potential influence associated with the later development of multiple ovulations. Comparisons were made between the subgroups with one follicle vs multiple follicles, using normalization to the beginning of deviation and to ovulation, as described for the group comparisons.

The correlation for LH concentration between the day of emergence and the day of the expected beginning of deviation was determined. This was done because of LH differences that approached significance between groups before deviation. In addition, repeatability of an end-point within each mare between consecutive postablation ovulatory waves was based on a significant correlation between the first and second postablation periods as previously described (Jacob et al. 2007a). Correlation coefficients for systemic concentrations of FSH and LH between consecutive ovulatory waves were determined for the days of ablation, emergence, expected deviation, ovulation, minimal concentration and maximal concentration. Discrete end-points for comparisons between

groups with one and multiple ovulations and for correlation analyses between waves within an animal were length of intervals from emergence of the follicle that produced the future ovulation to the expected beginning of deviation and from deviation to ovulation; number of follicles that attained a diameter of 6–9, 10–14, 15–19 or ≥ 20 mm by the end of the common growth phase; number of dominant follicles, ovulations and HAFs; diameter of F1, F2, F3 and F4 at the beginning of deviation; and diameter at maximum and on day −1 for the follicle that produced the ovulation.

Blood samples and hormone assays

Jugular blood samples were collected daily during two consecutive interovulatory intervals into heparinized tubes. Blood samples were centrifuged ($1500 \times g$ for 10 min) and decanted, and the plasma stored (−20°C) until assayed. Samples were assayed for FSH and LH by radioimmunoassay (Donadeu and Ginther 2002) and for oestradiol (Ginther et al. 2005a) and progesterone (Ginther et al. 2005b) by commercial kits, as validated and described for mare plasma in our laboratory. The intra- and interassay coefficients of variation (CV) and mean sensitivity were 9.2%, 18.4% and 1.1 ng/ml for FSH; 7.8%, 8.3% and 0.2 ng/ml for LH; 10.0%, 4.9% and 0.1 pg/ml for oestradiol; and 5.6% (intra-assay CV) and 0.04 ng/ml for progesterone, respectively.

Statistical analyses

Data for hormones were challenged for extreme values with the Dixon outlier test (Zar 1984). Data for end-points that were not normally distributed, according to Kolmogorov–Smirnov tests, were transformed to logarithms or ranks. Sequential data were analysed by SAS MIXED procedure with a REPEATED statement to account for autocorrelation between sequential measurements (Version 9.1.3; SAS Institute Inc., Cary, NC, USA). If a significant effect of day was detected, paired Student's *t*-tests were used to locate differences between selected days. Group (or subgroup) effect or interactions of day and group that were significant or approached significance were further examined by unpaired Student's *t*-tests within days. Correlations between the first and second postablation periods as an indication of repeatability for a specific day and end-point were carried out by the Spearman test. The Spearman correlation test was selected because it uses ranked data and is less affected by extreme values (Conover 1999). Single-point data were analysed by one-way ANOVA, and frequency data were analysed by chi-square tests of independence. A probability of $p \leq 0.05$ indicated that a difference was significant, and probabilities between $p > 0.05$ and $p \leq 0.1$ indicated that a difference approached significance. Data are given as mean ± SEM, unless otherwise stated.

Results

Comparisons of the first and second induced ovulatory waves did not indicate a significant difference for any of the characteristics, and data for the two waves were combined. All dominant follicles ovulated, except one, and comparisons were made between groups with one vs multiple ovulations; that is, a group was not available that had two dominant follicles and only one ovulation. The number of deleted waves and waves with various follicle outcomes are shown (Table 1). Variations in follicle outcomes and removal of waves for various reasons contributed to irregularities in number of observations among end-points (Table 2). Variation in numbers of follicles among end-points also resulted when F2, F3 or F4 were absent. Statistically outlying high LH concentrations or low progesterone concentrations resulted in the removal of two waves in the follicle and hormone analyses for the peri-ovulatory period.

The group with one ovulation had a subgroup of one follicle (F1; six waves) or an undersized F2 (7.6–14.9 mm; three waves) vs a subgroup of multiple follicles at the expected beginning of deviation. There was no subgroup effect or a subgroup-by-day interaction for diameter of F1 on days −7 to −1 relative to ovulation; only the day effect was significant (not shown). Comparisons of FSH concentrations showed significant main effects of subgroup and day and an interaction for 7 days before to 3 days after the beginning of deviation (Fig. 1). The interaction represented lower concentrations in the subgroup with multiple follicles for 2 days before to 2 days after deviation. There were no significant effects for FSH between subgroups for days −3 to 4 relative to ovulation (not shown). The day effect for LH normalized to deviation (Fig. 1) and normalized to ovulation (not shown) was significant, but the subgroup effect and interaction were not significant when normalized to either deviation or ovulation.

There were 32 of 46 (70%) waves with one dominant follicle and either one ovulation or an HAF. An additional wave in which one of two dominant follicles began to regress the day after reaching 28 mm was added to the group with one ovulation. The largest follicle (F1) at deviation in the group with one ovulation either ovulated (30 waves) or formed an HAF (two waves) in 32 of 33 (97%) waves. There were 28 dominant follicles in 13 of 46 (28%) waves with multiple ovulations and included 20 of 28 follicles that ovulated

Table 1. Relationships of follicle outcome to number of induced ovulatory waves with one or multiple ovulations

Outcome[a]	No. waves
Deleted, no dominant follicle	1
Deleted, no ovulation[b]	1
One ovulation[c]	
Determinable deviation	22
F2 unavailable	9
Obscured deviation	2
Multiple ovulations	
Determinable deviation	8
F3 unavailable	2
Obscured deviation	3
Total	48

[a]All dominant follicles ovulated or formed a haemorrhagic anovulatory follicle and were designated as an ovulation.
[b]Dominant follicle did not ovulate or form a haemorrhagic anovulatory follicle.
[c]Includes a wave in which the second-largest follicle began to regress the day after reaching 28mm.

Table 2. Mean ± SEM for comparisons of intervals and numbers and diameters of follicles for induced ovulatory waves between waves with one ovulation vs multiple ovulations and correlation coefficients between waves within individual mares[a]

End points	One ovulation	Multiple ovulations	Probability	Correlation between waves within a mare: Coefficient	Correlation between waves within a mare: Probability
Intervals (days)					
Ovulation to deviation[b]	16.7 ± 0.2 (33)	17.6 ± 0.4 (13)	$p < 0.02$	+0.10 (22)	NS
Ablation to emergence[c]	1.4 ± 0.1 (33)	2.5 ± 0.4 (13)	$p < 0.0001$	+0.20 (22)	NS
Emergence to deviation	5.4 ± 0.2 (33)	5.2 ± 0.2 (13)	NS	−0.04 (22)	NS
Deviation[b] to ovulation	6.2 ± 0.2 (32)	5.5 ± 0.3 (13)	$p < 0.08$	+0.45 (21)	$p < 0.04$
Ablation to ovulation	12.9 ± 0.3 (32)	13.2 ± 0.2 (13)	NS	+0.34 (21)	$p < 0.1$
Maximum diameter to ovulation	1.1 ± 0.1 (32)	1.2 ± 0.1 (13)	NS	−0.14 (21)	NS
No. of follicles at deviation[b]					
6–9 mm	2.3 ± 0.4 (33)	1.5 ± 0.6 (13)	$p < 0.10$	+0.53 (22)	$p < 0.02$
10–14 mm	2.5 ± 0.4 (33)	1.8 ± 0.5 (13)	NS	+0.44 (22)	$p < 0.05$
15–19 mm	1.9 ± 0.3 (33)	1.6 ± 0.4 (13)	NS	+0.26 (22)	NS
≥ 20 mm	1.3 ± 0.1 (33)	1.9 ± 0.4 (13)	$p < 0.001$	−0.33 (22)	NS
No. of dominant follicles	1.0 ± 0.0 (33)	2.2 ± 0.1 (13)	$p < 0.0001$	+0.18 (23)	NS
Diameters (mm)					
Beginning of deviation[d]					
Largest (F1)	22.8 ± 0.5 (22)	23.8 ± 0.5 (6)	NS	+0.64 (10)	$p < 0.05$
Second largest (F2)	20.5 ± 0.4 (22)	22.0 ± 0.6 (6)	$p < 0.06$	−0.53 (10)	NS
Third largest (F3)	17.8 ± 0.4 (22)	17.6 ± 0.8 (6)	NS	−0.51 (10)	NS
Fourth largest (F4)	15.8 ± 0.4 (20)	14.8 ± 0.7 (6)	NS	+0.18 (9)	NS
F1 minus F2	2.3 ± 0.5 (22)	1.9 ± 0.6 (6)	NS	+0.10 (8)	NS
Preovulatory follicle					
At maximum	39.5 ± 0.7 (32)	39.1 ± 1.4 (13)	NS	+0.46 (22)	$p < 0.04$
Day before ovulation	39.2 ± 0.7 (32)	38.5 ± 1.5 (13)	NS	+0.38 (22)	$p < 0.09$

[a]An ovulatory follicular wave was induced by ablation of follicles ≥ 6 mm 10 days after ovulation.
Dominant follicles either ovulated or formed a haemorrhagic anovulatory follicle and were designated as an ovulation.
[b]Expected beginning of deviation or the day when the largest follicle was closest to 22.6 mm. Number of follicles at deviation is the same as number during the common-growth phase.
[c]Emergence refers to the day the future ovulatory follicle was ≥ 6 mm.
[d]Determinable beginning of deviation.

and eight of 28 that formed an HAF. Eleven waves had two ovulations and two had three ovulations. Six waves produced two ovulations on the same day (three waves) or on consecutive days (three waves) with no associated HAFs. Seven waves produced ovulations and HAFs. The first follicle to ovulate or form an HAF originated from F1 (largest follicle at deviation) in nine of 13 (69%) waves with multiple ovulations. In the remaining waves, the first ovulation or HAF occurred from F2, but F1 also ovulated or formed an HAF.

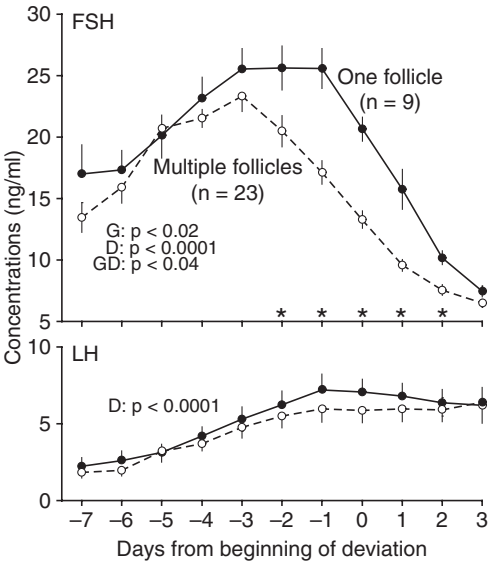

Fig. 1. Mean ± SEM for systemic concentrations of FSH and LH in induced ovulatory waves with subgroups of one vs multiple follicles ≥ 6 mm during the common growth phase in the group that developed one ovulatory follicle. The day of the beginning of expected deviation (largest follicle closest to 22.6 mm) was used for both subgroups, because of the absence of a usable F2 in the single follicle subgroup. Significant main effects and the interaction are shown. G = subgroup, D = day, GD = subgroup-by-day interaction. An asterisk indicates a day of a difference ($p < 0.05$) between subgroups

In the group with multiple ovulations (includes HAFs), an HAF began to form on the day before ovulation of the other follicle (one HAF), on the day of ovulation (three HAFs), or within 4 days after ovulation (four HAFs; mean day 1.3 ± 0.7). A dominant follicle formed an HAF more frequently ($p < 0.0008$) in the 13 waves with multiple ovulations (8/28 follicles) than in the waves with one ovulation (2/33). On the day before ovulation vs forming an HAF, the follicles did not differ significantly in diameter. Concentrations of FSH and LH were not significantly different between the waves with two ovulations vs waves with both ovulation and HAF formation, neither for data normalized to deviation nor for data normalized to the ovulation; only the day effects were significant (not shown).

The comparisons of single-point characteristics between waves with one vs multiple ovulations, using ovulatory terms in relation to either an ovulation or an HAF, are shown (Table 2). The intervals from ovulation to deviation and from ablation to emergence of the future ovulatory follicle were longer, and the interval from deviation to ovulation was shorter (approached significance) in the waves with multiple ovulations. The number of follicles at the end of the common growth phase or beginning of deviation in the ≥ 20-mm class

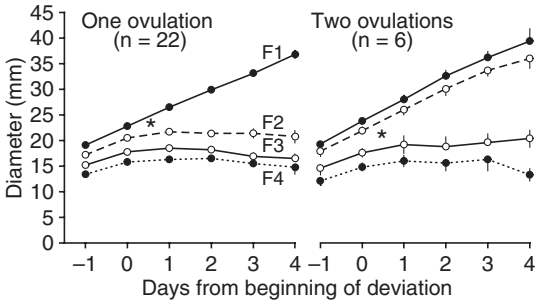

Fig. 2. Mean ± SEM for diameters of the four largest follicles (F1 to F4) before and after deviation in induced waves with determinable deviation and one vs two ovulations. Deviation occurred between F1 and F2 for waves with one ovulation and between F2 and F3 for waves with two ovulations. The asterisk indicates a difference ($p < 0.05$) between the indicated days in the diameter change for F1 vs F2 (one ovulation) and F2 vs F3 (two ovulations)

was greater in the waves with multiple ovulations, but the number in the other classes (6–9, 10–14 and 15–19 mm) did not differ between the waves with one vs multiple ovulations.

The diameter data profiles for F1, F2, F3 and F4 normalized to the beginning of determinable deviation are shown for waves with one and two ovulations (Fig. 2). Only six waves were available with two ovulations, because of two waves with three ovulations. Deviation occurred between F1 and F2 for waves with one ovulation and between F2 and F3 for waves with two ovulations, as shown (Fig. 2). The diameters of F1, F3 and F4 did not differ between waves with one vs two ovulations during the day before to 4 days after deviation (no main effects or an interaction; not shown), but the interaction was significant for F2. Diameters of F1, F2, F3 and F4 and F1 minus F2 on the day of determinable deviation did not differ between waves with one vs two ovulations (Table 2). Maximum diameter of the preovulatory follicle did not differ between wave groups.

There was no significant difference in systemic concentrations of FSH on the day of ablation between groups that developed one vs multiple ovulations during the ablation-induced ovulatory follicular wave. Concentrations of FSH showed a significant interaction for the period encompassing 7 days before to 3 days after the expected beginning of deviation; concentrations were lower on the day of deviation and for the 3 days after the beginning of deviation in the waves with multiple ovulations vs one ovulation (Fig. 3). From day −3 to day 4 relative to ovulation, FSH concentrations were lower in the waves with multiple ovulations, as indicated by a significant group effect. The interaction approached significance apparently from concentrations that were lower in the group with multiple ovulations on days −1 to 4 than on days −3 and −2.

Concentrations of LH on the day of ablation were higher ($p < 0.02$) in the waves that developed multiple ovulations (2.0 ± 0.5 ng/ml) than in the waves with one ovulation (1.2 ± 0.2 ng/ml). For LH concentrations during the interval from ovulation to ablation, the main effect of group approached significance ($p < 0.07$), because of a tendency for higher concentrations averaged over days in the group that developed multiple ovulations (6.4 ± 0.6 ng/ml) than in the group that developed one ovulation (5.5 ± 0.4 ng/ml). During 7 days before to 3 days after deviation, higher concentrations of LH for the waves with multiple ovulations approached significance (group effect) with significant differences during several days preceding deviation (Fig. 3). During days −3 to 4 relative to ovulation, the group-by-day interaction was significant, primarily from

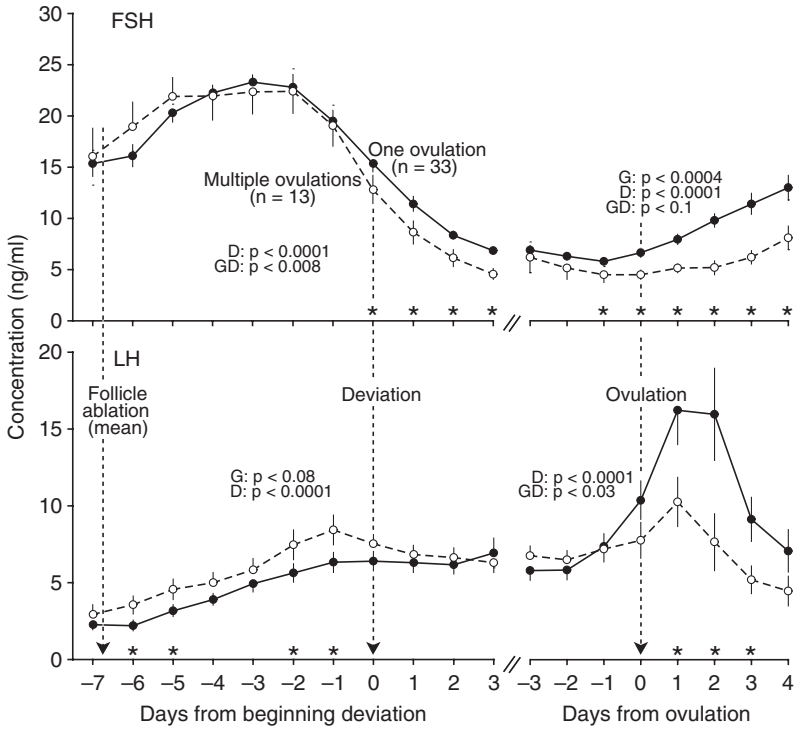

Fig. 3. Mean ± SEM for systemic concentrations of FSH and LH in induced ovulatory waves with one vs multiple ovulations. The formation of a haemorrhagic anovulatory follicle was included as an ovulation. Data were normalized to the beginning of deviation and to the first ovulation. Significance or approaching significance ($p < 0.1$) for main effects and the interaction are shown. G = group, D = day, GD = group-by-day interaction. An asterisk indicates a day of a difference ($p < 0.05$) between groups

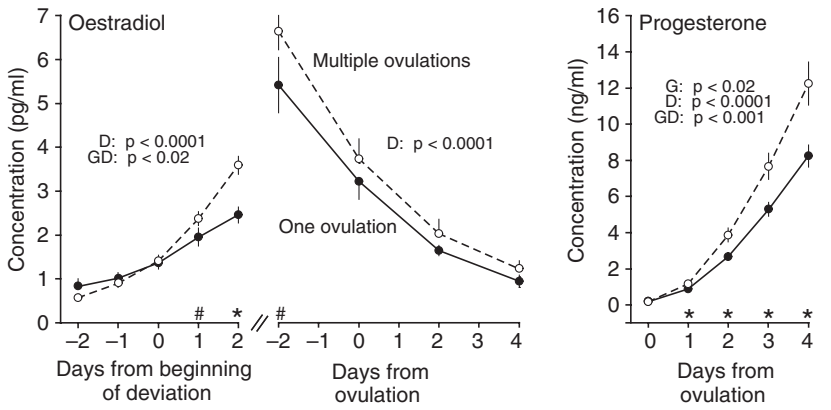

Fig. 4. Mean ± SEM for systemic concentrations of oestradiol and progesterone in induced ovulatory waves with one vs multiple ovulations. The formation of a haemorrhagic anovulatory follicle was included as an ovulation. Data were normalized to deviation and to the first ovulation. Number of waves for the one and multiple ovulations are 12 and 9 for oestradiol and 20 and 12 for progesterone, respectively. Significant main effects and the interaction are shown. G = group, D = Day, GD = group-by-day interaction. An asterisk indicates a day of a difference ($p < 0.05$), and a pound mark (#) indicates an approaching difference ($p < 0.07$) between groups

lower LH on days 1, 2, and 3 in the waves with multiple ovulations.

Oestradiol concentrations on the day of ablation did not differ significantly between waves that developed multiple ovulations (0.6 ± 0.1 pg/ml) vs one ovulation (0.8 ± 0.1 pg/ml). During 2 days before to 2 days after deviation, the effect of day was significant (Fig. 4). Averaged over the two groups, the first significant increase ($p < 0.0008$) occurred between 2 days before and 1 day before the beginning of deviation. An increase occurred between these 2 days in 86% of waves. The group-by-day interaction was significant, primarily because of higher concentrations ($p < 0.0003$) in the group with multiple ovulations 2 days after deviation and an approaching significant difference ($p < 0.07$) on the day after deviation. During days −2 to 4 relative to ovulation, oestradiol showed only a day effect from decreasing concentrations, although higher concentrations in the group with multiple ovulations approached significance ($p < 0.07$) on day −2.

The six means for concentrations of progesterone in the two groups with one and multiple ovulations on 2, 1, and 0 days before deviation ranged from 0.05 to 0.20 ng/ml, with no significant main effects or interaction (not shown). On days 0 to 4 relative to ovulation, the day effect and interaction for progesterone were significant (Fig. 4). In each of the 32 individual waves in the two groups, the progesterone concentration was higher on day 1 than on day 0. The interaction represented progressively higher ($p < 0.05$ to $p < 0.001$) concentrations in the group with multiple ovulations over days 1 to 4; concentrations were not different on day 0.

The correlation in systemic LH concentrations between the day of ablation and the day of deviation was positive and significant ($r = +0.52$, $p < 0.0003$). The correlations between waves within mares, indicating within-animal repeatability, were positive and significant for the interval from deviation to ovulation, approached significance for the interval from ablation to ovulation, significant for number of follicles at the end of the common growth phase in the 6–9 and 10–14 mm classes,

Table 3. Correlation coefficients for systemic concentrations of FSH and LH on specific days between consecutive ovulatory waves within 22 individual mares, indicating repeatability

	FSH		LH	
On day of:	Coefficient	Probability (p)	Coefficient	Probability (p)
ablation	+0.58	<0.005	+0.44	<0.05
emergence	+0.60	<0.003	+0.71	<0.0002
beginning of deviation	+0.59	<0.004	+0.84	<0.0001
ovulation	+0.44	<0.04	+0.64	<0.002
minimal concentration	+0.70	<0.0002	+0.41	<0.06
maximal concentration	+0.72	<0.0001	+0.71	<0.0003

significant for maximum diameter of the follicle preceding ovulation, and approached significance for diameter on the day before ovulation (Table 2). When waves with only one ovulation were considered, there were stronger and significant correlations for diameter of the preovulatory follicle at maximum ($r = +0.70$, $p < 0.01$) and on the day before ovulation ($r = +0.66$, $p < 0.02$). Concentrations of FSH and LH each showed a significant and positive correlation between waves within animals on the days of ablation, emergence, deviation, ovulation, at minimal value and at maximal value, except that the correlation for LH at minimal value only approached significance (Table 3). The repeatability or correlation between consecutive waves for number of dominant follicles was not significant. The incidence of multiple ovulations during the second induced wave was not significantly different between mares that had multiple ovulations during the first induced wave (3/6) and mares that did not (4/17).

Discussion

In the group with one dominant follicle, the subgroup with only one follicle (F1) or one follicle and an undersized F2 during the common growth phase was less effective than the subgroup with multiple follicles in reducing the FSH concentrations preceding the

beginning of deviation. This was indicated by the 2-day earlier beginning of a decline in FSH and lower concentrations of FSH during the 2 days before the beginning of deviation in the subgroup with multiple follicles. The relationships of follicle number to the extent of suppression of FSH during the common-growth phase has also been demonstrated by experimental manipulation of follicle number in mares (Donadeu and Ginther 2001). Similarly, lower FSH was associated with more follicles in the follicular wave in cattle (Haughian et al. 2004). Although the unavailability of an inhibin antigen precluded assay of inhibin in the present study, the enhanced FSH suppressing effect of multiple follicles can be attributed to greater inhibin output on the basis of a report involving experimental manipulation of number of follicles in mares; it was concluded that the first 2 days of the predeviation FSH decrease was caused by inhibin (Donadeu and Ginther 2001).

The depiction of determinable deviation associated with one vs two ovulations showed deviation between F1 and F2 for the one-ovulation group and between F2 and F3 for the two-ovulation group. In a study of spontaneous waves, 11 of 32 (34%) had two dominant follicles but only one wave had two ovulations; therefore, the nature of deviation when two dominant follicles ovulate was not considered (Jacob et al. 2007b). In the present study of induced ovulatory waves, a similar number (14/46; 30%) had multiple dominant follicles, but in marked contrast to the results reported for spontaneous waves, all but one of 29 dominant follicles in the multiple-dominant waves ovulated (20 follicles) or formed an HAF (eight follicles). It appears that the induction of ovulatory waves by follicle ablation and treatment with PGF2α did not alter the incidence of multiple dominant follicles, but greatly increased the incidence of multiple ovulations by the multiple dominant follicles. Thus, the ablation/PGF2α technique apparently produced a model for using multiple ovulations to study the relationships between ovulation and hormone concentrations in ponies. However, confirmation of ablation-induced stimulation of multiple ovulations will be needed, given that contemporary controls were not available in the present experiment. Regardless, the number of multiple ovulations (including HAFs) was adequate for study of the hormone/ovulation dynamics by comparisons between groups with one and multiple ovulations.

The approximately 1-day later emergence of the future first ovulatory follicle in the waves that developed multiple vs one ovulation accounted for an approximately 1-day longer interval from the ablation to deviation. However, the relationship of the later emergence to the development of multiple ovulations is unknown and requires confirmation. The only detected difference in numbers and diameters of follicles in the group that later developed multiple ovulations was more follicles ≥ 20 mm at the beginning of deviation. Other differences only approached significance, but included a larger F2, which is compatible with more follicles ≥ 20 mm. In a study of spontaneous waves, greater diameter of F2 in a group that developed two vs one dominant follicle also approached significance (Jacob et al. 2007b).

Concentrations of FSH at ablation and during the common growth phase before the beginning of deviation were similar between waves that later developed one vs multiple ovulations. The predeviation decrease in FSH and increase in oestradiol occurred synchronously in both groups, without a difference between groups for either hormone. A systemic oestradiol increase before the beginning of deviation has been reported previously (Gastal et al. 1999c; Bergfelt et al. 2001). The present FSH/oestradiol temporal relationships are consistent with reports of an FSH-suppressing role for inhibin throughout the FSH decline encompassing deviation (Donadeu and Ginther 2001) and an additional negative effect of oestradiol as deviation approaches (Bergfelt et al. 2001). Both oestradiol and a proteinaceous fraction of follicular fluid either alone or synergistically suppressed circulating concentrations of FSH (Miller et al. 1979; Bergfelt and Ginther 1986). FSH was lower at and after deviation in the waves with multiple ovulations. Correspondingly, oestradiol began to diverge between groups beginning at deviation and reached significantly higher levels within 2 days after deviation. In a reported study, concentration of FSH was lower in spontaneous waves with two dominant follicles but only one ovulation, beginning the day before deviation (Jacob et al. 2007b). However, oestradiol was not different between groups that developed one vs two dominant follicles in contrast to the present results in induced waves. Thus, an apparent hormonal difference between induced and spontaneous waves that may be related to the more frequent conversion of multiple dominant follicles into multiple ovulations was the greater post-deviation oestradiol production by the multiple future dominant and ovulatory follicles in induced waves when compared with the reported concentrations in spontaneous waves.

Concentrations of LH were greater (approached significance) during the interval between ovulation and ablation, were significantly greater on the day of ablation, and were greater (approached significance) during the common growth phase in the ablation-induced waves that developed multiple dominant follicles and multiple ovulations. Greater LH concentrations in some waves throughout the common growth phase were consistent with the positive and significant correlation in LH concentrations within waves between the day of ablation and the day of the beginning of deviation. These results indicated that multiple ovulations of dominant follicles may be more common in mares with higher concentrations of LH. Concentrations of LH had a measurable degree of repeatability between consecutive waves in this and a previous study (Jacob et al. 2007a). Experimental reduction of LH encompassing deviation in mares resulted in lower concentrations of oestradiol (Bergfelt et al. 2001). These findings are compatible with the concept that higher LH in certain mares preceding deviation favors the development of multiple oestrogen-competent follicles ≥ 20 mm at deviation, leading to the development of multiple ovulations. This is consistent with the report that LH was not elevated until 3 days after the beginning of deviation in spontaneous waves that developed anovulatory double dominant follicles (Jacob et al. 2007b). However, given that LH differences before

deviation only approached significance in the present study, the indications for early LH involvement in development of multiple ovulatory follicles in mares requires further study.

Ablation of follicles and administration of PGF2α 10 days after ovulation resulted in an immediate increase in LH concentrations, followed by a plateau beginning at deviation, as previously reported (Gastal et al. 1999a, 2000). In a project involving ablations on day 10 as in the present study, but without administration of PGF2α in a control group, an LH increase did not begin until day 15 or 3 days before the beginning of deviation (Gastal et al. 2000). Our interpretation is that the post-ablation LH increase in the present study resulted from the reduction in progesterone from the PGF2α treatment. Cessation of the LH increase in both groups at the beginning of deviation likely represented the LH-inhibiting effect of the follicles (Gastal et al. 1999b; Ginther et al. 2005b) by the production of oestradiol; oestradiol has been shown to have an inhibitory effect on LH throughout the ovulatory LH surge (Ginther et al. 2007b). In spontaneous waves, LH does not begin to increase until just before deviation (Jacob et al. 2007a), when the naturally decreasing progesterone reaches a concentration that is inadequate for a negative effect (Ginther et al. 2006b, 2007a).

Several hormone differences associated with one vs multiple ovulations were detected during the peri-ovulatory period. The lower FSH before day 0 (day of ovulation) may have reflected the higher (approached significance on day −2) oestradiol concentrations associated with multiple preovulatory follicles. Although an inhibin assay was not available for the present study, the relatively greater depression of FSH after multiple ovulations than after one ovulation may have resulted from discharge of follicular fluid and inhibin from multiple follicles into the peritoneal cavity, as described for single ovulations (Nambo et al. 2002). In this regard, a dose of progesterone that decreased the LH concentrations in mares did not affect FSH concentrations (Gastal et al. 1999a). Earlier studies on progesterone/FSH relationships produced equivocal results (Ginther 1992).

Another pronounced effect was the reduction in LH after ovulation (days 1 to 4) in the group with multiple ovulations. The lower LH likely reflected, at least in part, the greater concentrations of progesterone on the corresponding days. Previous reports have documented higher concentrations of progesterone during dioestrus in mares that had multiple ovulations (Henry et al. 1982; Urwin and Allen 1983; Squires et al. 1987) but did not indicate that the higher progesterone in double-ovulating mares begins immediately after the ovulations. Support for the interpretation that the lower post-ovulation LH in the group with multiple ovulations results from higher progesterone is the demonstration of a negative effect of exogenous progesterone on LH (Gastal et al. 1999a, 2000). In addition, temporal relationships between progesterone and LH in single-ovulating mares indicate that the LH-reducing negative effect of progesterone begins after LH concentrations reach maximum on day 1 (Ginther et al. 2006b, 2007a).

A previous study of repeatability by correlations within individual mares in consecutive spontaneous waves (Jacob et al. 2007a) did not consider events or diameters during the common growth phase, because of follicle overlapping from previous waves. The present study minimized overlapping, using follicle ablation with induction of a new wave. During the common growth phase, no significant within-animal correlations as indications of repeatability were found for length of intervals between follicle events, but repeatability was detected in the number of small follicles (6–9 mm and 10–14 mm) in the ovulatory wave during the common-growth phase. The number of larger follicles (> 14 mm) and number of dominant follicles were not significantly correlated between consecutive waves. In this regard, an increased frequency of double ovulations between the first and second wave was not detected in mares that had a double ovulation during the first wave. However, this can be attributed to the small number of mares, considering the many reports of repeatability of double ovulations in surveys with large numbers of mares (reviewed in Ginther 1992). The reported correlations in maximal diameter of the preovulatory follicle found within mares for consecutive spontaneous waves (Jacob et al. 2007a) was also found for consecutive induced waves in the present study. The potential importance of this finding to equine breeding programs has been discussed (Jacob et al. 2007a). The significant correlations as indicators of repeatability in concentrations of FSH and LH found at specific events throughout the induced ovulatory waves in the present study is consistent with the previously reported repeatability on many days during the oestrous cycle (Jacob et al. 2007a). Repeatability within mares in both LH concentrations and double ovulations is compatible with the concept that mares with relatively high concentrations of LH are more likely to develop double ovulations.

In conclusion, ovulatory follicular waves were induced by ablation of follicles and administration of PGF2α 10 days after ovulation. The presence of only one follicle or an undersized second largest follicle during the common growth phase was not as effective as multiple follicles in reducing the predeviation concentrations of FSH. The incidence of multiple dominant follicles (≥ 28 mm) in the ablation-induced waves was 30%, and all but one of the multiple dominant follicles ovulated (20 follicles) or formed an HAF (8 follicles). The only detected nonhormonal characteristics that preceded the development of multiple dominant follicles that ovulated or formed an HAF were later follicle emergence and more follicles ≥ 20 mm at the end of the common-growth phase or beginning of deviation. In the group with multiple ovulations or HAFs, LH concentrations were higher preceding deviation (approached significance), FSH concentrations were lesser on the day of deviation and thereafter, and oestradiol concentrations became greater soon after deviation. Apparently, more follicles ≥ 20 mm resulted in more circulatory oestradiol which speculatively may have been associated with the ability of the follicles to later ovulate or form an HAF. During the peri-ovulatory period, differences between one and multiple ovulations or HAFs were consistent with negative effects of the ovarian hormones on the gonadotropins.

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