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Conversion of a viable preovulatory follicle into a hemorrhagic anovulatory follicle in mares

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Abstract

Formation of hemorrhagic anovulatory follicles (HAFs) was studied daily in pony mares, beginning before ovulation (controls, n = 7) or HAF formation (n = 7). Solitary HAFs were used that were not accompanied by an ovulation from another follicle during the late follicular phase. The day of ovulation and the day of detection of excessive specks floating in the antrum were designated Day 0 for the controls and HAF group, respectively. There were no significant differences between groups in concentrations of systemic progesterone, LH, or FSH before Day 3, but estradiol was higher ($P < 0.05$) in the HAF group than in the controls on Day -3. The incidence in the controls of discrete ultrasonographic follicle indicators of impending ovulation (decreased turgidity, loss of spherical shape, echoic specks in antrum, serration of granulosum, and an apical area) was greater ($P < 0.007$) for Day -1 (2.3 ± 0.6 indicators/mare) than for Day -2 (0.1 ± 0.1), showing a temporal relationship to impending ovulation. There was no difference between groups in preovulatory indicators on either day. These results, as well as similar diameter between groups on Day -1, indicated that HAF formation occurred in a follicle that did not have altered B-mode ultrasonographic structure before the day of expected ovulation. Furthermore, the follicle cells were viable, as indicated by luteinization of the wall of the HAF; there were no differences between groups in circulating progesterone concentrations during Days 0 to 17. The percentage of follicle circumference with color-Doppler signals indicating blood flow on Day - 1 was greater ($P < 0.03$) in the HAF group (90 ± 4%) than in the controls (69 ± 7%). Results indicated that elevated plasma estradiol a few days before expected ovulation and greater vascularity of the follicle on the day before expected ovulation were associated with the conversion of a viable follicle into an HAF.

Keywords: anovulation, follicles, gonadotropins, ovarian steroids, mares.

Introduction

When blood enters the antrum of an equine preovulatory follicle which subsequently fails to ovulate, the resulting structure has been termed a hemorrhagic anovulatory follicle (HAF), based on gray-scale ultrasound (Ginther and Pierson, 1984b; 1989) and on a previous description of blood oozing from the cut surface (Ginther, 1979). Structures similar in description have also been termed hemorrhagic follicles (Ginther, 1979; 1992; Ginther and Pierson, 1989), anovulatory hemorrhagic follicles (Carnevale *et al.*, 1989), and persistent anovulatory follicles (McCue and Squires, 2002). What may have been the same structures were noted originally by transrectal palpation toward the end of the ovulatory season (Burkhardt, 1948). In a report on ovarian estrogen levels, similar structures were termed autumn follicles (Knudsen and Weiert, 1961). The economic importance of HAFs as a breeding-management problem in mares has been noted (McCue and Squires, 2002; McKinnon, 1998; Pycock, 2000) and reflects anovulation of a follicle after the mare has been bred.

An early indication of a developing HAF is an excessive number of floating echoic specks in the follicular fluid, as indicated by ballottement during ultrasonographic examination (Ginther, 1995b). The first day that the number of the floating specks during HAF formation exceeds the expected number for a preovulatory follicle seems equivalent to the day of expected ovulation, but this has not been shown critically. Reported descriptions indicate that strand-like echoic lines form a network one or two days after appearance of the specks (reviewed in Ginther, 1995b). Follicular fluid of mares contains a heparin-like anticoagulant (Stangroom and Weevers, 1962). It has been suggested that the ultrasonically determined developmental changes of HAFs result from gradually decreasing effectiveness of the follicular-fluid anticoagulant (Ginther, 1992), but this has not been demonstrated critically.

The temporal associations between circulating concentrations of hormones and HAF formation have not been reported, except in one mare with apparently normal LH and FSH concentrations (Ginther, 1979). Sequential circulating progesterone concentrations have not been reported in mares with HAFs or compared between mares with and without HAFs. It has been noted, however, that some mares with HAFs had concentrations below 1 ng/ml and others had concentrations that reached above 1 ng/ml (Carnevale *et al.,* 1989;

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McCue and Squires, 2002). In conclusion, the concentrations of circulating hormones before and during HAF development are unknown. In regard to gonadotropins, HAFs form in both nontreated and hCG-treated mares (Carnevale *et al.,* 1989; McCue and Squires, 2002), and similar structures form during pregnancy when mares are producing the gonadotropic hormone, eCG (Ginther, 1992).

In one study, HAFs were detected by ultrasound in 4.7% of 213 interovulatory intervals. An HAF occurred without a companion ovulation or a second HAF during the late follicular phase (five mares), in conjunction with ovulation of another follicle (one mare) or during the luteal phase (three mares; Ginther and Pierson, 1989). Other reported incidences have extended from 5% to 23% of estrous cycles (Gastal *et al.,* 1998; McCue and Squires, 2002). The HAFs were more common late in the year and in old mares and tended to occur repeatedly in individuals. Structures that seem similar in description to HAFs in horses also have been detected during ultrasonographic examinations in llamas (Adams *et al.*, 1991) and women (Pierson and Chizen, 1994), but information on etiology or associated physiology is not available. Therefore, study of HAF formation and the underlying mechanisms in horses may be of comparative importance for other species, including humans. In addition, the anovulation associated with HAF formation may be a useful natural model for studies on the mechanism of ovulation and luteal development.

The purpose of the present study was: 1) to characterize the changing ultrasonographic morphology of HAFs that develop in follicles that were expected to ovulate; 2) to establish a common time-related ultrasonographic reference point between ovulation and the beginning of HAF formation, so that the associated physiology can be compared between control and HAF groups; 3) to search for ultrasonographic structural signs in the wall of the preovulatory follicle that distinguish between impending ovulation versus impending HAF formation; and 4) to determine if HAF development involves alterations in profiles of systemic hormones (progesterone, estradiol, LH, FSH) before and after the beginning of formation.

Materials and Methods

Animals

Animals were handled in accordance with the United States Department of Agriculture Guide for Care and Use of Agricultural Animals in Research. Nonlactating pony mares of mixed breeds, 4–16 yr of age, weighing 320–490 kg, and with docile temperament were used in the Northern Hemisphere (43° N). Feed consisted of alfalfa/grass hay with access to water and trace-mineralized salt. The score for body condition for all mares was high throughout the experiment (score ≥ 7; Henneke *et al.,* 1983). An early onset of the ovulatory season was induced by a lighting program (Ginther, 1992), so that the ovulatory season began in February and March, rather than in April and May. Thereafter, mares were kept under natural light.

Ultrasonography

To generate optimal ultrasound images, mares were lightly sedated during scanning with a low dose of detomidine hydrochloride (1 mg per animal, i.v.; Dormosedan, Pfizer Animal Health, Philadelphia, PA, USA). A duplex B-mode (gray-scale) and pulsed-wave color-Doppler ultrasound instrument (Aloka SSD-2000; Aloka America, Wallingford, CT, USA) equipped with a finger-mounted 7.5-MHz convex-array transducer (UST-995-7.5) was used for transrectal scanning. The principles, techniques, and interpretation of B-mode (Ginther, 1995a) and color-flow mode (Ginther and Utt, 2004) examination of the mare reproductive tract have been reviewed. The color-flow mode was used to display blood flow in vessels of the follicle and HAF; the spectral mode was not used. All Doppler scans were performed at a constant color-gain setting, a velocity setting of 10 cm/sec, and a filter setting of 4. The velocity and filter settings were used to minimize detection of venous flow and extraneous movement. However, the effect of these settings on detection of venous flow in the tissues of this study is unknown. The entire follicle was scanned in a slow continuous motion several times. Real-time B-mode/color-flow images from a digital video camera were used for evaluation of some of the end points in the laboratory.

Experiment 1

Six mares with a history of HAFs during two or more estrous cycles (repeaters) during the ovulatory season of the previous year and six mares with no history of HAFs (controls) were selected. On average, the repeaters had HAFs during 3.5 of 10.2 estrous cycles per mare during the previous year, whereas the controls had no HAFs during 8.6 estrous cycles. This portion of the experiment was done during April and May. Ovaries were scanned daily beginning 12 days after ovulation by B-mode ultrasonography until a ≥ 25 mm follicle developed. Thereafter, ovaries were scanned by B-mode and a blood sample was taken daily from a jugular vein until ovulation or HAF formation during the late follicular phase. Examinations with the color-Doppler mode began when the follicle was ≥ 35 mm. The late follicular phase was defined retrospectively as continued growth of the follicle after ≥ 25 mm and development of an estrus-like echotexture of the endometrial folds (Ginther and Pierson, 1984a) or a progesterone concentration of < 1 ng/ml for ≥ 3 days, regardless of whether the preovulatory-sized follicle

ovulated or formed an HAF. Controls had a solitary dominant follicle. Mares in the HAF group had a solitary HAF that developed from the dominant follicle during the late follicular phase without ovulation from another follicle during the period and did not form an HAF or ultrasonically detectable luteinization of follicles during the luteal phase that followed the experimental late follicular phase. This was done so that the hormonal comparisons between groups were not complicated by other HAFs or ovulations. Both ovulation and the first day of HAF formation were designated Day 0. The day of the beginning of HAF formation was assigned retrospectively as the first day of consecutive daily appearances of excessive numbers (too numerous to count) of echoic specks, imparting a cloudy appearance to the follicular fluid. In the absence of the detection of excessive specks, the day before echoic strands or an echoic sheet appeared on a two-dimensional image of the antrum was used as Day 0.

After Day 0, scanning by B-mode was done daily until Day 8 and then on Days 10, 12, 14, and 17 and by color-flow mode daily until Day 5. Blood samples were taken daily until Day 5 and then on Days 7, 14, and 17. In the HAF repeater mares, the scanning and sampling schedule were done during two estrous-cycle equivalents. The first cycle with an appropriate HAF was used in the analyses. However, in one mare the schedule was also followed during a third cycle, owing to the development of HAFs that did not meet the appropriate criteria until the third cycle. An appropriate HAF occurred during the late follicular phase in four mares. Therefore, four mares were selected by randomization from the control mares for comparisons with the four HAF mares.

In addition to the four HAF mares obtained from the HAF repeaters during the first portion of the experiment, three mares were used that developed an HAF during September and October during an independent study with a different objective (unpublished). The mares ovulated at the end of study, indicating they had not entered the anovulatory season. The three mares from September and October (second portion of experiment) and the four from April and May (first portion) were from the same herd during a continuous 12-month period; each of the seven mares was used only once. The HAFs from the September/October group met the criteria established for the April/May group. The three HAF mares were not used in the previous independent study, and the follicle/HAF records and blood samples were assigned to the HAF group of the present study. Three mares that did not develop an HAF in the same independent study and with no known history of HAFs were randomly selected as controls. There were no significant differences ($P < 0.05$) for any of the end points between the four mares with HAFs in April and May and the three with HAFs in September and October. Therefore, mares were combined for group comparisons, yielding seven mares in a control group with a solitary ovulation and seven mares in an HAF group with an appropriate solitary HAF during the late follicular phase.

End points

Diameters of the preovulatory follicle, the follicle that formed the HAF, and the HAF were obtained from the average of height and width of the antrum (follicles) or the entire structure (HAF), using the apparent maximal area from each of two frozen images. When the HAF exceeded the width of the image field, a split screen was used. The echotexture of the endometrium was scored 1 to 4 (minimal to maximal edema of the endometrial folds); scores of 3 and 4 were considered representative of estrus (Ginther and Pierson, 1984a). The score on Day 0 and the highest score on Days -3 to 0 were used for comparison between groups. Discrete (nonquantitative) B-mode characteristics of the preovulatory follicle that have been reported to indicate impending ovulation (Pierson and Ginther, 1985; Carnevale *et al.,* 1988; Townson and Ginther, 1989; Gastal *et al*., 2006b) were recorded as present or absent for both groups on Days -2 and -1. The discrete end points were: 1) decreased turgidity of the follicle under transducer pressure, 2) loss of spherical shape, 3) echoic spots floating in the antrum, and 4) an apical area or cone-shaped protrusion of the follicle (future ovulation site). In addition, we have observed that both surfaces of the granulosum (interfaces with the antrum and with the theca interna) become irregular (serration of granulosum) as ovulation approaches (Gastal *et al*., 2006b), and presence or absence of serration also was recorded. The number of discrete signs/mare for Days -2 and -1 was compared between the two groups. In addition, turgidity, shape, and serration of granulosum were also assessed for Days 0 to 5 in the HAF group.

For color-flow mode, the percentage of circumference of the follicle wall with an apparent network of vessels was estimated from the blood-flow color displays during the real-time scanning. Percentage of circumference of the follicle wall was estimated from real-time images of the sequential two-dimensional planes during scanning of the entire follicle. Therefore, the term circumference refers to the periphery of a three-dimensional structure. The transducer was held at various angles to obtain maximal color signals of the follicular wall; angle between the ultrasound beam and blood flow affects the detectability and extent of the color signals (Zwiebel and Pellerito, 2005). The technique for estimating the percentage of the wall of an equine follicle with color-Doppler signals has been reported (Gastal *et al.,* 2006a). A similar approach has been used in women (Chui *et al.,* 1997; Bhal *et al.,* 1999; Coulam *et al.,* 1999).

Experiment 2

This was a confirmatory experiment and was done to clarify an equivocal result of high plasma estradiol concentrations a few days before expected ovulation in the HAF group of the primary experiment. Five of the six mares with a history of HAFs the previous year were monitored daily by ultrasonography for two estrous-cycle equivalents during June and July. In addition, five mares with a history of no HAFs were monitored for one estrous cycle. A blood sample was taken for estradiol assay when the largest follicle reached ≥ 30 mm. Ultrasound scanning was done daily thereafter as described for Experiment 1 to determine if the follicle ovulated or formed an HAF.

Blood samples and hormone assays

Jugular blood samples were collected in the morning into heparinized tubes in control and HAF groups. Samples were centrifuged (1500 x g for 20 min), decanted, and stored (-20º C) until assay. Samples were assayed for progesterone and LH during Day -4 to Day 17 and for estradiol and FSH on Day -4 to Day 5. Plasma samples were assayed for FSH and LH by radioimmunoassay as validated and described for mares in our laboratory (Donadeu and Ginther, 2002). The intra- and inter-assay CVs and mean sensitivity, respectively, were 8.1%, 10.9%, and 0.6 ng/ml for FSH and 7.3%, 3.5%, and 0.1 ng/ml for LH. Plasma concentrations of estradiol were measured by a double-antibody radioimmunoassay kit (Double Antibody Estradiol, Diagnostic Products Corporation, Los Angeles, CA, USA) as described and validated in our laboratory for mare plasma (Ginther *et al.,* 2005a). The intra- and inter-assay CVs were 9.3% and 12.1%, respectively, and sensitivity was 0.1 pg/ml. Plasma progesterone concentrations were determined using a solid-phase radioimmunoassay kit containing antibody-coated tubes and ^{125}I-labeled progesterone (Coat-A-Count Progesterone, Diagnostic Products Corporation, Los Angeles, CA, USA) as described and validated in our laboratory for mare plasma (Ginther *et al.,* 2005c). The intra-assay CV and the sensitivity were 6.2% and 0.04 ng/ml, respectively.

Statistical analyses

Data for each hormone were not normally distributed and were transformed to natural logarithms. Analyses of hormone concentrations and B-mode characteristics were done by the SAS MIXED procedure (version 8.2; SAS Institute Inc., Cary, NC) to determine the main effects of group and day and their interaction, using a repeated statement to account for the autocorrelation between measurements. Unpaired *t*-tests were used to locate differences between groups when a significant or an approaching significant group effect or interaction was obtained. Estradiol data from Experiment 2 were analyzed by ANOVA. Analyses of color-Doppler data on Day -1 were examined by ANOVA; number of observations was too small on other days, owing to the beginning of examinations at ≥ 35 mm. Frequency data were evaluated by chi-square tests. A probability of $P \leq 0.05$ indicated that a difference was significant and a probability between $P > 0.05$ and $P \leq 0.1$ was considered as approaching significance. Data are presented as the mean ± SEM, unless otherwise indicated.

Results

Two of the six mares with a history of HAFs during the previous year ovulated at the end of both follicular phases in the present study. A solitary HAF appropriate to the study occurred during one of the late follicular phases in each of the four remaining mares. The incidence of an appropriate HAF was 23% (4 of 13 periods) in the mares with an HAF history. The total incidence of HAFs of all types, considering multiple HAFs as one event, was 54% (7 of 13 estrous cycles). The three HAF events that were not considered for the comparisons were multiple HAFs during the follicular phase, an HAF and an ovulation during the same follicular phase, and HAFs during a luteal phase. In the mares without a history of HAFs, the incidence was 0% (0 of 6 cycles). In the independent study, an appropriate HAF occurred in three of 38 (8%) estrous cycles or mares; no other HAFs occurred during the study.

The length of the interval between ovulation or HAF formation and the next ovulation or HAF formation (equivalent to an interovulatory interval) was not different between the seven controls (22.2 ± 0.9 days) and the seven mares in the HAF group (22.5 ± 0.8 days). Endometrial score was not different between the HAF and control groups on Day 0 (3.4 ± 0.2 and 3.6 ± 0.2, respectively) or for the highest score on Days -3 to 0 (3.8 ± 0.2 and 3.9 ± 0.0). Diameter of the preovulatory follicle over Days -4 to -1 was not different between the control and HAF groups (Fig. 1). Diameter of the HAFs increased from a mean of 40 mm on Day 1 to a mean of 59 mm on Day 3 and then gradually decreased to 26 mm by Day 17 (day effect, $P < 0.001$). The largest HAFs reached a maximum of 80 mm in two of the seven mares.

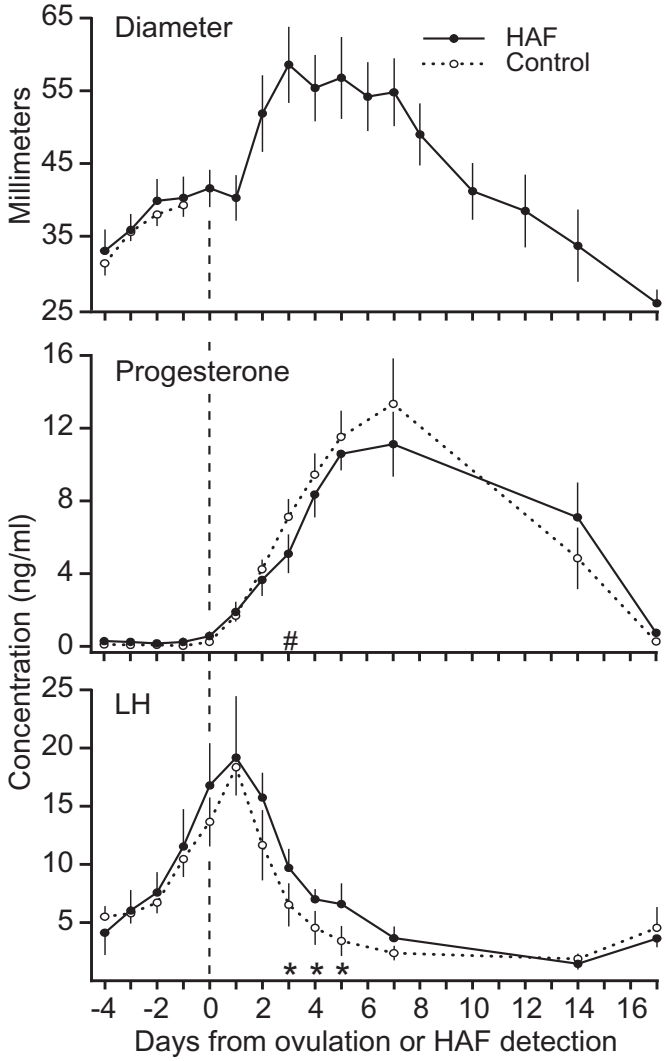

Figure 1. Means ± S.E.M. for diameters of the preovulatory follicle before ovulating or forming a hemorrhagic anovulatory follicle (HAF) and after formation of an HAF and for progesterone and LH concentrations in the control and HAF groups (n = 7 mares/group). Diameter of the preovulatory follicle was not different between groups on Days -4 to -1. The day effect was significant ($P < 0.0001$) for progesterone, but the group effect and the interaction of day and group were not. The day effect ($P < 0.0001$) and interaction ($P < 0.003$) were significant for LH, but the group effect was not. A pound mark (#) indicates approaching significance and an asterisk (*) indicates significance between groups for the indicated days.

For the factorial analysis of number of discrete indicators of impending ovulation, only the group effect was significant with more indicators on Day -1 than on Day -2 (Table 1). The frequencies of each individual indicator on Day -1 were not different ($P > 0.1$) between groups. The total number of indicators/mare on Day -1 for the two groups was as follows: decreased turgidity (11 of 14 mares, 78%), loss of spherical shape (36%), echoic specks in antrum (36%), serration of granulosum (28%), and development of an apical area (28%). In the HAF group, decreased turgidity, loss of spherical shape, and serration of granulosum were present in all mares on Day 0, in five of seven mares on Day 1, and in one mare on Day 2. None of the indicators were detected, thereafter. A rent or disruption in the HAF wall was detected in four of seven mares in the HAF group, primarily on Days 2 to 4, but a rent in the follicle was not detected in any control or HAF mare before Day 0 or in any HAF mare on Day 0.

Table 1. Mean ± SEM for number of discrete indicators[a] of impending ovulation.

	Group (n = 7 mares / group)[c]	
Day[b]	Control	HAF
–2	0.1 ± 0.1	0.8 ± 0.5
–1	2.3 ± 0.6	2.0 ± 0.7

[a]Decreased turgidity, loss of spherical shape, echoic spots in antrum, apical area, and serration of granulosum.
[b]Day 0 = day of ovulation or first day of HAF formation.
[c]Day effect, P < 0.002; group effect, NS; interaction, NS; NS = not significant.

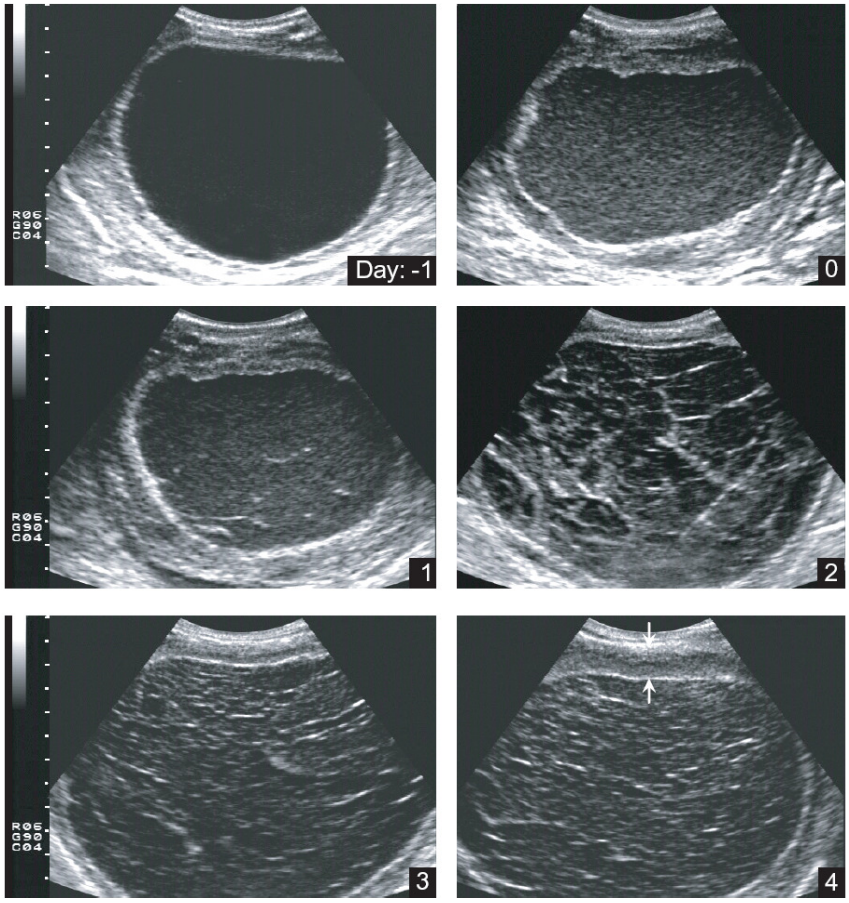

Figure 2. B-mode ultrasonograms taken sequentially of the same structure, illustrating the most common events in formation of an HAF. Day -1 (equivalent to the day before ovulation): indistinguishable by B-mode from a preovulatory follicle on Day -1. Day 0 (equivalent to the day of ovulation): excessive floating specks in antrum. Day 1: floating specks and the beginning of echoic bands. Day 2: network of bands that quivered when HAF was ballotted. Day 3: maximum diameter with contents firm upon ballottement. Day 4: increased thickness of a wall of apparent luteal tissue delineated by apposing arrows. Distance between two adjacent graduation marks is 5 mm (left margin).

The changing characteristics of HAFs in five of seven mares were as follows: excessive echoic specks in the follicular fluid on Day 0 or echoic strands in the antrum on Day 1; quivering of a network of strands upon ballottement on Day 2; and firmness upon ballottement on Day 3 (Fig. 2), except that firmness was first detected on Day 2 in one mare and on Day 4 in another. Pockets of floating specks were detectable among the echoic strands on Day 1. In the remaining two of seven mares, a two-dimensional image of an echoic sheet, rather than strands, appeared on the images for Day 1, and an excessive number of specks was present in the remainder of the structure for several days (Fig. 3). The two-dimensional sheets represented a pool of sediment as indicated by real-time scanning for a three-dimensional perspective with periodic

ballottement. On Day 0 (day before strand or pool formation), specks were not detected (one mare), were similar to the extent of specks in some mares on the day before ovulation (two mares), or were excessive (four mares). The network of strands was detectable in the center of the HAF even when the HAF had reduced to the mean diameter of 26 mm on Day 17 (Fig. 1).

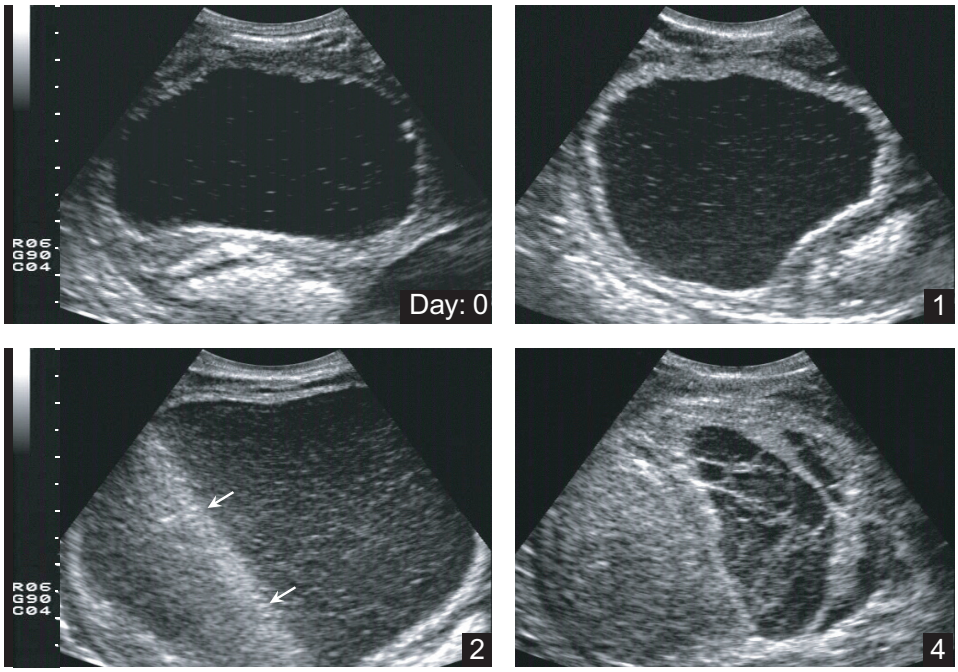

Figure 3. B-mode ultrasonograms taken sequentially of the same structure, illustrating an alternate sequence in HAF formation. Day 0 (equivalent to the day of ovulation): excessive specks in antrum. Day 1: thickening of granulosa apparently in association with beginning of luteinization; the echoic mass causing an indentation in the HAF wall is an HAF from the previous cycle. Day 2: pool of apparent sedimentation to the left and floating echoic specks to the right; the interface between the sedimentation pool and floating specks is indicated by arrows. Day 4: firm structure with a network of echoic bands in the former area of floating specks. Distance between two adjacent graduation marks is 5 mm (left margin).

The estimated percentage of the follicle wall on Day -1 with color-Doppler signals was greater ($P < 0.03$) for the HAF group (89.6 ± 4.2%) than for the controls (69.3 ± 7.4%; Fig. 4). Color-Doppler signals were detected in the wall of the apical area in each of two mares with an apical area in the HAF group but not in the two mares with an apical area in the controls.

Concentrations of progesterone ($P < 0.0001$) during Days -4 to 17 (Fig. 1) and FSH during Days -4 to 5 ($P < 0.001$; Fig. 5) showed a day effect for each hormone but no group effect or interaction of group and day. Although there was no interaction for progesterone, the concentrations approached significance ($P < 0.07$) being lower in the HAF group on Day 3. Concentrations of LH (Fig. 1) showed a day effect ($P < 0.0001$) and an interaction ($P < 0.003$); concentrations were higher in the HAF group on Days 3 ($P < 0.05$), 4 ($P < 0.03$), and 5 ($P < 0.02$). The day effect was significant ($P < 0.0001$) for estradiol during Days -4 to 5, the group effect approached significance ($P < 0.1$), and the interaction was not significant (Fig. 5). According to *t*-tests, concentrations were higher in the HAF group than in the controls on Day -4 ($P < 0.06$) and Day -3 ($P < 0.05$). The development of a luteinized wall of HAFs (Fig. 2), based on echotexture, was associated with extensive color-Doppler signals of blood flow (Fig. 4).

In the confirmatory study (Experiment 2), the preovulatory 30-mm follicle formed an HAF during three of 10 late follicular phases in mares with a previous history of HAFs. The follicle ovulated during the remaining 12 follicular phases, combined for the two groups. Plasma estradiol concentration was higher (group effect; $P < 0.0001$) in three phases with an HAF (4.7 ± 0.8 pg/ml) than in the remaining seven phases with an ovulation in mares with a history of HAFs (2.2 ± 0.3 pg/ml) or in the five phases in mares with no history of HAFs (1.8 ± 0.3 pg/ml).

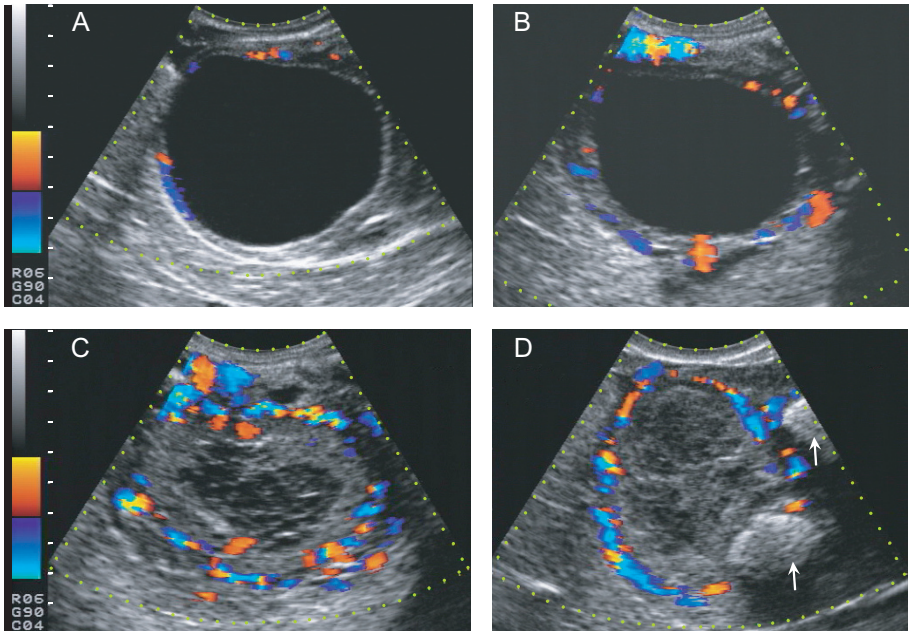

Figure 4. Ultrasonograms from different mares with color-Doppler signals in the wall of a follicle or HAF. A: preovulatory follicle on Day -1 or the day before ovulation. B: follicle on day before the beginning of HAF formation. C: HAF on Day 6 with color signals in the outer wall. D: HAF on Day 8; the two echoic structures (arrows) with shadowing artifacts beneath are remnants of HAFs from the previous cycle. Distance between two adjacent graduation marks is 5 mm (left margin).

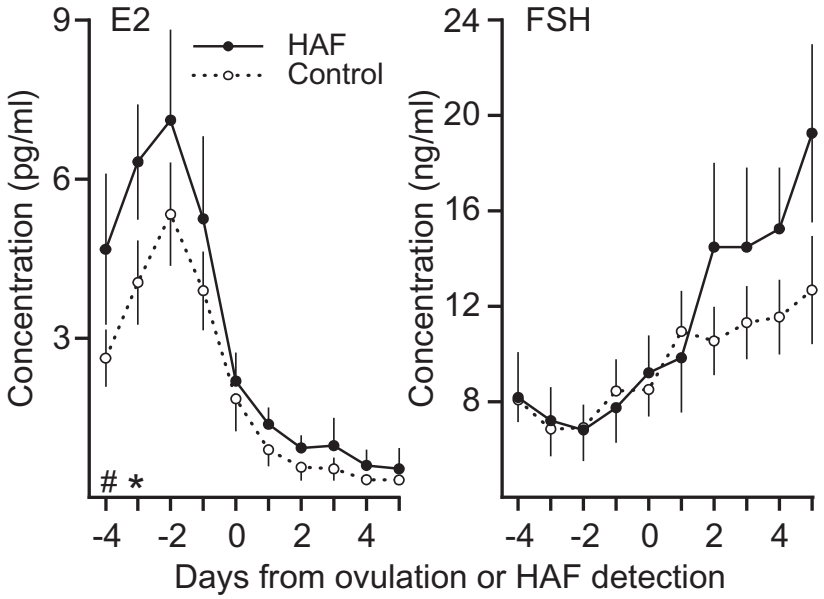

Figure 5. Means ± S.E.M. for concentrations of estradiol and FSH in the control and HAF groups (n = 7 mares/group). For each end point the day effect was significant ($P < 0.001$) but the interaction was not. The group effect approached significance ($P < 0.1$) for estradiol and was not significant for FSH. A pound mark (#) indicates approaching significance and an asterisk (*) indicates significance between groups for the indicated days.

Discussion

These are apparently the first reported experiments specifically designed to compare ovulation with HAF formation in any species. The studies are of comparative importance because of the lack of information on similar structures in llamas (Adams *et al*., 1991) and women (Pierson and Chizen, 1994). The repeatability of HAF formation in mares with a history of HAFs during the previous year was demonstrated by the 31% incidence for HAFs appropriate to the study and 54% incidence for HAFs of all types. In comparison, no HAFs occurred in mares with a history of no HAFs. This repeatability result confirms clinical observations (see Introduction).

The incidence of discrete follicle structural changes (decreased turgidity, loss of spherical shape, echoic specks in antrum, serration of granulosum, and an apical area) provided an indication of impending ovulation, as demonstrated by increased frequency between Day -2 and Day -1. Decreased follicle turgidity, as shown by applying pressure with the transducer, was more than twice as frequent as the other discrete signs on Day -1. Although serration of granulosum and echoic specks in the antrum occurred less frequently, they were the most reliable indicators in that none were detected on Day -2. Assessment of discrete indicators of impending ovulation every 24 hours was not useful in predicting whether an ovulatory-sized follicle would ovulate or form an HAF, as indicated by the lack of a group effect or a group-by-day interaction for number of indicators/mare. Follicle diameter also was not different between groups on Day -1. The ultrasonographic B-mode results indicated that HAF formation involved the apparent entry of blood into a follicle, based on echotexture of the structure. Reported descriptions of the gross appearance of HAFs upon sectioning of excised ovaries are those of a blood clot (Knudsen and Weiert, 1961; Ginther, 1979).

The percentage of circumference of the follicle with color-flow signals on Day -1, indicating blood flow, was greater in the HAF group. However, this did not appear to be a reliable indicator of an impending HAF in an individual because of overlapping values between groups. The increased proportion of the follicle wall with color-flow signals on Day -1 in mares with a future HAF was partly attributable to vessels in the apical area in two of two pre-HAF follicles but not in two of two ovulatory follicles. Detection of an apical area in only two of seven mares in each group is attributable to the limitations of daily examinations and an interval of < 1 day between formation of an apical area and ovulation. The apical area represents the future point of rupture during ovulation (Pierson and Ginther, 1985). The decreased blood flow in the apical area of a follicle before ovulation has been reported previously in humans (Brannstrom *et al.,* 1998), but not in horses. The absence or presence of detected blood flow in the apical area of a future ovulation or HAF formation, respectively, is compatible with minimal hemorrhage during ovulation and gradual but massive hemorrhage during HAF formation.

The most common (five of seven mares) daily sequential ultrasonographic morphology on Days 0 to 3 of a solitary HAF originating in the late follicular phase involved the formation of excessive follicular-fluid echoic specks, echoic strands, quivering of the network of strands upon ballottement, and firmness upon ballottement, respectively. The network of strands on the two-dimensional images presumably were from fibrin septae that develop in blood clots (McGorum *et al.*, 1996). The HAFs increased in diameter about 38% between Days 1 and 3, reaching maximum diameter on the day that firmness was first detected. The increasing diameter presumably was from blood continuing to enter the structure, but this was not determined, directly. The source of the blood that entered the follicle initially and apparently continued to enter the HAF is not known. The initial source may be from incomplete ovulatory rupture, but disruptions or tears were not detected at the beginning of HAF formation. Resolution of this aspect of HAF formation may require more frequent examinations (e.g., hourly). In this regard, evacuation of antrum in the ovulatory process occurs in about 1 min in most mares (Townson and Ginther, 1987). Tears in the HAF wall were noted in some mares at about the time that the HAF was increasing in diameter. It is not known whether the tears disrupted vessels and allowed additional blood to enter the HAF. Hematoma contents appeared to protrude externally through some of the apparent tears, forming small sacs less than 10 mm in diameter.

The remaining two of the seven solitary HAFs that formed during the late follicular phase did not form a network of strands, initially. Instead, an echoic pool was evident and involved about 25 to 75% of the maximal two-dimensional image of the HAF, depending upon the plane of view. Most likely, the pool was from gravitational settling of red blood cells or aggregates of primarily red cells to the bottom of the follicle. This was indicated by a relatively smooth surface of the pool and a location ventral or opposite to the dorsal attachment of the ovarian vascular pedicle. Our interpretation is that these two HAFs formed in the presence of a higher concentration or effectiveness of an anticoagulant than for the five HAFs that became firm within a few days. This would account for the apparent settling of red blood cells or aggregates of red cells and a delay in the organization of the supernatant (plasma and follicular fluid). This is the first report of extensive differences in ultrasonographic morphology among HAFs. It seems reasonable that the presence of heparin-like components of follicular fluid (Stangroom and Weevers, 1962) and their half-life contributed to the sequential changes seen on the ultrasonic images within structures and to the

variation among structures.

The HAFs that were multiple or formed during the luteal phase were similar in ultrasonographic morphology to the HAFs that formed during the late follicular phase. The HAFs that formed during the luteal phase originated from the dominant follicles of a major secondary follicular wave. The development of a major anovulatory wave before the emergence of the ovulatory wave occurs in about 25% of estrous cycles (Ginther *et al.,* 2004). In the previous studies, formation of HAFs from such waves was not reported. Further study is needed on the pathogenesis of HAFs that form during the follicular phase versus the luteal phase.

Endometrial score was not different between groups for the 3 days before ovulation. All individuals had an estrus-like endometrial echotexture (score 3 or 4), except one mare in the HAF group. The increase in circulating progesterone concentrations between Days -1 and 0 in both the ovulating control group and the HAF group is consistent with results of a study in ovulating mares (Townson *et al.,* 1989). The progesterone and LH similarities between groups before and at the designated Day 0 indicated that the ultrasonographic characteristics used to define the beginning of an HAF were reasonable. Formation of an HAF versus ovulation was not attributable to differences in progesterone or LH concentrations, based on the common Day 0 reference point. In this regard, formation of solitary HAFs, multiple HAFs, both an HAF and an ovulation during a late follicular phase, and HAFs during a luteal phase demonstrated that HAFs form during diverse hormonal milieus.

The preovulatory estradiol surge in mares reaches a peak 2 days before ovulation or 3 days before the peak of the LH surge (Ginther *et al.,* 2005b). A preovulatory FSH increase began on the day estradiol began to decrease; this temporal hormonal relationship has not been previously reported. A similar LH profile occurred in the present study in both groups. A group effect that approached significance and a *t*-test that was significant provided a tentative indication that estradiol concentrations were higher in the HAF group on Day -3 or before peak concentrations. This equivocal result was clarified, however, in the confirmatory Experiment 2. Plasma estradiol concentrations were higher in an HAF group than in an ovulatory group when the solitary follicle was $\geq$ 30 mm. Actual diameter was a mean of 31.6 mm and occurred on mean Day -3.4 which is similar to the day when estradiol concentrations appeared to be higher in the primary experiment. Thus, elevated plasma estradiol occurred in the HAF group during the late follicular phase before the peak of the estradiol surge, but not at the peak or in the two-day interval between the peak and initial HAF formation. The apparent role of elevated estradiol in HAF formation a few days later is unknown. Further study beginning earlier in the estrous cycle will be required, including the assessment of vascular development and changing concentrations of follicular-fluid factors (e.g., other steroids, cytokines, prostaglandins, growth factors, angiogenic factors).

The tentative indication that progesterone concentrations were lower on Day 3 and the higher LH concentrations during luteal development on Days 3–5 in the HAF group are likely interrelated and a reflection of the reported negative effect of progesterone on LH (Gastal *et al.,* 1999; Ginther *et al.,* 2005b). The well vascularized apparently luteinized wall of the HAFs (Fig. 4) is consistent with the similarity in progesterone production between the two groups. Luteinization of the follicle cells indicates that the follicle was healthy at the initiation of HAF formation. The similarity between groups in the length of the luteal phase, as indicated by the progesterone profiles, is consistent with the similarity in length of the interovulatory intervals. Progesterone production was similar between the corpus luteum of controls and the luteinized wall of an HAF, indicating that the extensive folding of the wall at follicle evacuation is not necessary for functional development of a corpus luteum. Although there were no significant differences in progesterone output between groups in this study, nil or minimal wall thickening or luteinization (Ginther, 1979) and progesterone production (Carnevale *et al.,* 1989; McCue and Squires, 2002) have been observed in some individual HAFs (incidence unknown).

In conclusion, sequential daily changes in the formation of most HAFs involved excessive specks floating in the antrum, echoic strands traversing the structure, and gel-like quivering followed by firmness of the hematoma upon ballottement. In other HAFs, apparent gravitational settling of red blood cells occurred and specks and later strands appeared in the fluid above the pool of apparent red cells. The variation in the concentrations and half-life of reported heparin-like components of the follicular fluid likely contributed to the sequential changes within a structure and to the variation among structures. Follicle viability was supported by the similarity in gray-scale images between the ovulating controls and the HAF group in diameter and discrete B-mode structural changes on the day before both ovulation and the beginning of HAF formation. The viability of the cells of a follicle that formed an HAF also was indicated by conversion of the follicle cells to luteal cells with an effective level of progesterone output. The progesterone and LH concentrations were similar between the ovulating and HAF groups, before and at the day of ovulation or initiation of anovulation, respectively. Higher estradiol concentrations occurred a few days before HAF formation, but other systemic hormones (progesterone, LH, FSH) were not altered during the conversion of a preovulatory follicle into an HAF. The percentage of follicle circumference with blood flow (color-flow signals) on Day -1 was greater in the HAF group than in the controls. Results indicated that elevated

concentrations of systemic estradiol a few days before expected ovulation and greater vascularity of the follicle on the day before expected ovulation were involved in the conversion of a viable preovulatory follicle into an HAF.

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