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Relationship between vascularity of the preovulatory follicle and establishment of pregnancy in mares

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Abstract

The relationship between follicle vascularity and establishment of pregnancy was studied in 21 mares. An ovulation-inducing injection of hCG was given when the preovulatory follicle was 34.0 to 37.0 mm (Hour 0). Mares that had not ovulated by 30 h after treatment were bred once (Hour 30). Each mare ovulated by 48 h after treatment, and 14 mares became pregnant and 7 were nonpregnant. The preovulatory follicle was evaluated by B-mode and Doppler ultrasonography at Hour 0 (before treatment) and Hour 30 (before breeding). B-mode echogenicity and thickness of the stratum granulosum and prominence of the anechoic band beneath the granulosum increased in both pregnant and nonpregnant groups (hour effect, $P < 0.001$) with no group effect or interaction. An increase in follicle diameter and percentage of follicle circumference with color-Doppler signals was greater between Hours 0 and 30 in the pregnant group than in the nonpregnant group (interactions, $P<0.001$). Spectral-Doppler measurements were made at the most prominent intraovarian color signal. Decreases in resistance and pulsatility indices were greater between Hours 0 and 30 in the pregnant group than in the nonpregnant group (interactions, $P < 0.05$), indicating increased vascular perfusion downstream from the spectral measurement in the pregnant group. Relative peak systolic velocity and time-averaged maximum velocity of blood flow at the point of spectral assessment showed a group effect ($P < 0.05$; greater in pregnant group) without an hour effect or interaction. Results supported the hypothesis that greater blood flow to the preovulatory follicle is associated with higher pregnancy rate.

Keywords: blood flow, follicle, mares, pregnancy rate, preovulatory follicle.

Introduction

Limited information is available in farm species on the effect of the characteristics of the preovulatory follicle on subsequent pregnancy rate. In cattle, GnRH-induced ovulation of follicles ≤ 11 mm resulted in decreased pregnancy rates in association with decreased circulating estradiol on the day of insemination and lower rate of progesterone increase after insemination (Perry *et al.*, 2005).These results indicated that induction of ovulation before the follicle is physiologically mature has a negative impact on establishment of pregnancy. Diameter of the preovulatory follicle did not affect pregnancy rate when ovulation occurred spontaneously. Induction of ovulation is common in mares, especially with hCG (reviewed in Ginther, 1992). The use of hCG does not have an adverse effect on pregnancy rate, indicating that only physiologically mature follicles respond to hCG stimulation. The effect of characteristics of the preovulatory follicle on the pregnancy-producing capacity of the oocyte has been studied in women, owing to the popularity of assisted-reproduction programs. The extent of the follicular wall with color-Doppler detected blood flow was positively associated with pregnancy rate (Chui *et al.*, 1997; Bhal *et al.*, 2001; Borini *et al.*, 2001). There was no significant relationship between pregnancy rate and uterine artery or intraovarian Doppler pulsatility index (Bhal *et al.*, 2001). A recent in vitro fertilization study in women found that well-vascularized follicles early in a follicular wave and on the day of hCG treatment late in the wave resulted in a higher pregnancy rate after embryo transfer (Shrestha *et al.*, 2006). Results of another study indicated that examining vascular impedance distal to an intraovarian artery by spectral Doppler indices may be useful in assessing the quality of an oocyte (Du *et al.*, 2006).

Despite the increasing and productive use of the transvaginal Doppler technology for assessing the quality of oocytes by the blood-flow characteristics of the follicle in assisted-reproduction programs in women, similar studies apparently have not been done in association with spontaneous ovulation. In farm species, oocyte and embryo collection have become common techniques (Betteridge, 2006), but the relationship between follicle vascularity and oocyte competence in establishment of pregnancy, with or without oocyte or embryo collection, has not been reported.

Transrectal Doppler ultrasonography is beginning to be used in mares for studying other reproductive mechanisms (Bollwein *et al.*, 2002; Acosta *et al.*, 2004; Silva and Ginther, 2006). Changes in echotexture and color-Doppler signals of the follicle

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wall as ovulation approaches in mares with and without hCG treatment have also been studied (Gastal *et al.*, 2006). An increase in granulosa thickness and echogenicity and a decrease in circulating estradiol during the 36 h post-treatment was greater in the hCG group than in the controls. Percentage of follicle wall with color-flow signals and prominence of the signals increased in both groups during the 36 h. During the 4 h before ovulation, the two groups showed similar decreases in prominence and percentage of wall with an anechoic band and prominence and percentage of wall with color-flow signals. The anechoic band is located in the area of the thecal layers and its characteristics have been described (Gastal *et al.*, 1998; 1999). The morphology accounting for the changes in the anechoic band and the increasing echogenicity of the granulosa layer as ovulation approaches have been discussed (Gastal *et al.*, 2006).

Luteal progesterone is essential for various mechanisms associated with early pregnancy, but it is not clear whether a deficiency in luteal development or progesterone production can result in a natural reduced pregnancy rate (reviewed in Ginther, 1992; Sevinga *et al.* 1999). Conflicting results on primary luteal insufficiency as a cause of failure of pregnancy establishment have been obtained in horses. An effect of defective vascularization of the corpus luteum on reduced pregnancy rate apparently has not been considered in any species.

The Doppler technology generates many end points concerning blood-flow velocities and patterns, but the ovarian artery of mares usually does not accommodate the placement of an angle cursor for the determination of velocities (Ginther and Utt, 2004). However, relative, as apposed to actual, velocities between experimental groups or side of body during an arterial pulse can be obtained without the angle cursor. Indices of vascular impedance in the tissues beyond the point of velocity assessment in the ovarian artery or its branches are especially useful end points because the indices are independent of angle of insonation (angle at intersection between direction of blood flow and direction of the ultrasound beam; Ginther and Utt, 2004; Zwiebel and Pellerito, 2005). These end points may substantiate the results of direct assessment of the extent of blood-flow signals but were not used in the reported study of hCG-induced changes in equine preovulatory follicles (Gastal *et al.*, 2006).

The hypothesis for the present study in mares was that a higher pregnancy rate is associated with greater blood flow to the preovulatory follicle.

Materials and Methods

Animals

Animals were handled in accordance with the United States Department of Agriculture Guide for the Care and Use of Animals in Agricultural Research. Nonlactating pony mares (n=26) of mixed breeds, 5 to 20 yr of age, and weighing 260 to 450 kg were used. The experiment was done from June 15 to September 15 in the Northern Hemisphere, and all mares ovulated after the experiment was completed. 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; body condition for all mares was high throughout the experiment. Mares with docile temperament and no apparent abnormalities of the reproductive tract as determined by ultrasound examinations (Ginther, 1995) were selected 15 d after ovulation. Mares were not used that developed a second follicle $\geq$ 27 mm, ovulated before breeding, were nonreceptive to natural breeding, or developed uterine fluid (> 3 mm in height) 48 h after breeding.

Gray-scale ultrasonographic examinations were done daily, beginning 15 d after the previous ovulation. When the largest follicle was 34.0 to 37.0 mm, all selected mares were given a single i.v. injection of 2500 IU of hCG (Hour 0). Scanning was done for ovulation detection 30 h after hCG treatment (Hour 30). Mares that had not ovulated by Hour 30 were scanned by Doppler ultrasound and then were bred. Mares were scanned at 18 h after breeding to check for ovulation and at 48 h to check for uterine fluid; even small collections of uterine fluid are associated with uterine inflammation and reduced pregnancy rate (Ginther *et al.*, 1985; Adams *et al.*, 1987; Cadario *et al.*, 1999). The next scanning was done 7 and 13 d after ovulation for evaluation of the corpus luteum and for pregnancy diagnosis, respectively. Blood samples for hormone assay were collected at Hours 0 and 30 and 7 d after ovulation.

Natural breeding 30 h after hCG treatment was done once by one of two stallions, designated Stallions A and B. The assignment of a stallion to a mare was done by randomization for every replicate of two mares, and only the assigned stallion was used. Successful breeding was based on intromission with tail flagging and confirmed by digital detection of ejaculatory pulses.

Doppler ultrasonography

To generate optimal ultrasound images, mares were sedated during scanning with a subdose of detomidine hydrochloride (1 mg per animal, i.v.; Dormosedan, Pfizer Animal Health, Philadelphia, PA, USA). A duplex B-mode 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. For B-mode, the brightness and contrast controls of the monitor and the gain controls of the scanner were standardized to constant settings (Gastal *et al.*, 1998). The color mode was used to display signals of blood flow in vessels of the follicle wall. For maximal flow-velocity detection by color signals without aliasing, the velocity range was set at 10 cm/s. The color-gain setting

was kept constant. The B-mode and color-Doppler end points were evaluated while the entire follicle was being scanned in a slow continuous motion several times.

For the spectral-Doppler mode, the setting for the range of flow-velocity detection was adjusted in each scanning to obtain the optimal spectral graph. The sample cursor or gate was set at a width of 1 mm. The angle of insonation was unknown, and therefore the velocities were not absolute but were considered relative in the comparisons for hours and groups (Zwiebel and Pellerito, 2005). The gate was placed at an intraovarian location with the most prominent color signal. A Doppler spectrum with three cardiac cycles was generated, and one of the cycles was used for spectral measurements. This also was done a second time, and the mean of the two measurements was used in the statistical analyses.

End points

Follicle diameter was obtained from the average of height and width of the antrum at the apparent maximal area from two frozen images. Percentage of circumference of the follicle wall with an anechoic band (Fig. 1) was estimated from B-mode 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 follicle. Other B-mode end points (prominence of anechoic band, echogenicity and thickness of granulosa) also were evaluated during real-time scanning and were scored from 1 to 3 (minimal to maximal) as described (Gastal *et al.*, 2006). Scores were assigned without reference to the score from the previous examination. The scores were made from the real-time images encompassing the entire follicle to minimize the impact of localized areas of the follicle wall that were obscured by ultrasound artifacts. Only Operator A scanned for the B-mode end points but Operators A and B independently estimated values from the monitor. These two operators were experienced and used these end points for a previous study (Gastal *et al.*, 2006). The validity of the scoring approach for mares has been demonstrated (Ginther, 1995).

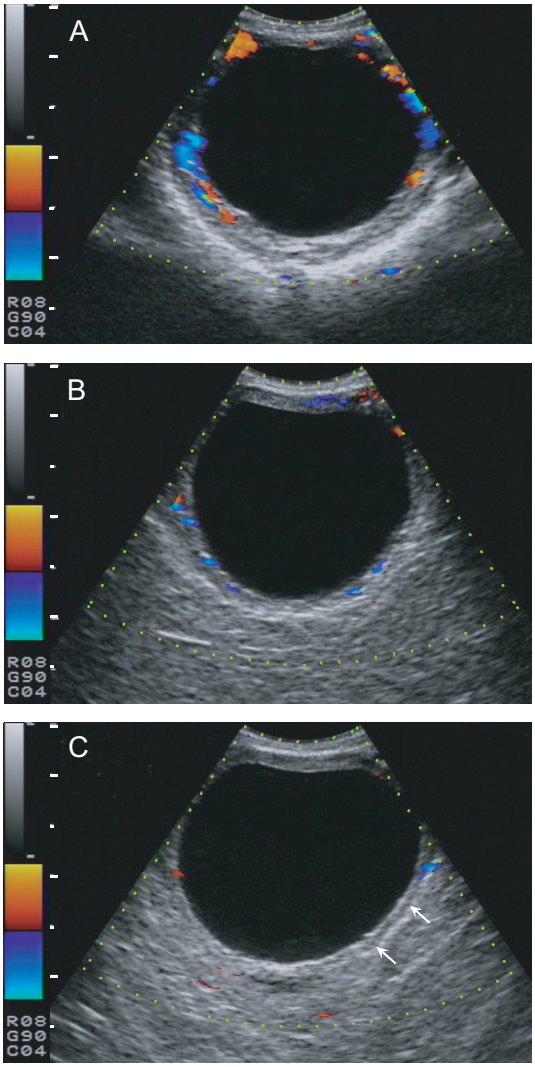

Figure 1. Color-Doppler sonograms illustrating blood-flow signals in the wall of preovulatory follicles of a mare (A) that became pregnant and a mare (two images in different planes; B,C) that did not become pregnant. Images were taken 30 hours after hCG treatment and show high (A) and low (B,C) percentages of follicular wall with blood-flow signals. A portion of an anechoic band is shown (arrows, C). Distance between graduation marks is 10 mm (left margin).

For color Doppler-mode, the percentage of circumference of the follicle wall with an apparent network of arterioles was estimated from the blood-flow color displays (Fig. 1) of the real-time sequential two-dimensional planes of the entire follicle. The transducer was held at various angles to display the maximum overall color signals throughout the three-dimensional circumference of the follicular wall. Estimations of the proportion of the follicle circumference with blood flow signals also have been used in women (Bhal *et al.*, 1999; Chui *et al.*, 1997; Coulam *et al.*, 1999). The percentage approach for the equine preovulatory follicle has produced similar results by two operators working independently (Gastal *et al.*, 2006). Prominence of the color displays was based on intensity of color and extent of coverage and was scored from 1 to 4 (minimal to maximal). Values for the two color-signal end points were estimated independently by two experienced operators during scanning by one of the operators. The spectral evaluations were done separately by a third operator. The end points for the gated intraovarian color signals were resistance index (RI), pulsatility index (PI), peak systolic velocity (PSV), and time-averaged maximum velocity (TAMV). The meaning and formulas for these spectral end points are well established (Zwiebel and Pellerito, 2005).

The corpus luteum 7 d after ovulation was compared between pregnant and nonpregnant groups for cross-sectional area (cm^2), estimated percentage of the entire gland with blood-flow signals, and the four spectral end points (RI, PI, PSV, TAMV) from the most prominent intraovarian color signal. The validity of using the estimated percentage of the structure with blood-flow signals for evaluation of the equine corpus luteum has been documented by comparison to the number of colored spots and pixels in frozen images (Ginther *et al.*, 2006).

Blood samples and hormone assays

Blood samples were collected into heparinized tubes and centrifuged (1500 x g for 20 min), and the plasma was decanted and stored (-20 °C) until assay. Plasma samples were assayed for estradiol and progesterone. The estradiol assay concentrations used a double-antibody radioimmunoassay kit (Double Antibody Estradiol, Diagnostic Products Corporation, Los Angeles, USA), as described and validated in our laboratory for mare plasma (Ginther *et al.*, 2005b). The intra-assay CV and sensitivity were 2.4% and 0.2 pg/ml, respectively. Plasma progesterone concentrations were measured 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.*, 2005a). The intra-assay CV and sensitivity, respectively, were 13.2% and 0.03 ng/ml.

Statistical analyses

The values for the end points in B-mode and color mode obtained independently by two operators did not show a statistical main effect of operator or an operator-by-group interaction, and the mean value for the two operators was used in the analyses. Quantitative data were analyzed by the SAS MIXED procedure to determine the main effects of group (pregnant and nonpregnant) and hour (Hours 0 and 30) and their interactions, using a repeated statement to account for the autocorrelation between measurements (version 8.2; SAS Institute Inc., Cary, NC). Measurements for the corpus luteum 7 d after ovulation were analyzed by a one-way ANOVA. A probability of $P < 0.05$ indicated that a difference was significant and a probability between $P > 0.05$ and ≤ 0.1 indicated that significance was approached. Data are presented as the mean ± SEM, unless otherwise indicated.

Results

The number of mares that were removed from the experiment after having been preselected was as follows: 1) presence of a second follicle ≥ 27 mm, 0 mares; ovulated before 30 h after hCG treatment, 2 mares; nonreceptive to breeding, 1 mare; and uterine fluid 48 h after breeding, 2 mares. The number of mares remaining for the experiment was 21; 10 were bred by Stallion A and 11 were bred by Stallion B; only one mare was available for the last replicate. All of the 21 mares ovulated between 30 and 48 h after hCG treatment, and breeding by the designated stallion was successful for each mare. The pregnancy rate was greater ($P < 0.03$) for Stallion A (9 of 10) than for Stallion B (5 of 11). Age of mare and the interval between use of a stallion for breeding were not different between the pregnant group (9.0 ± 1.1 yr; 5.6 ± 0.8 d) and the nonpregnant group (10.2 ± 1.6 yr; 4.4 ± 0.9 d).

Data for B-mode (Fig. 2) and color-flow mode for the preovulatory follicle and spectral mode for the most prominent color-flow signal (Fig. 2) in the ovary at Hour 0 (hCG treatment) and Hour 30 (breeding) and significant main effects and interactions are shown. A mean increase between Hour 0 and Hour 30 averaged over the two groups (hour effect), without a group-by-hour interaction, was obtained for granulosa echogenicity and thickness and for prominence of the anechoic band and prominence of color-flow signals. A group-by-hour interaction was obtained for follicle diameter and percentage of circumference with color-

flow signals; the interaction reflected primarily greater values at Hour 30 in the pregnant group. When the Doppler sample gate was placed on an intraovarian color-flow signal, an interaction was obtained for RI and PI reflecting lower values in the pregnant group at Hour 30. A significant group effect from higher values in the pregnant group, but without an interaction, was obtained for PSV and TAMV. Plasma estradiol concentration decreased in both groups after treatment with hCG (Fig. 3), but there were no significant differences for progesterone (not shown).

There were no significant differences 7 d after ovulation between the pregnant group and nonpregnant group for area of the corpus luteum (7.7 ± 1.0 vs 6.3 ± 0.9 cm^2), percentage of corpus luteum with blood-flow signals (56.1 ± 4.1 vs 58.8 ± 5.9 %) or prominence of signals (3.2 ± 0.1 vs 3.1 ± 0.1 score), RI (0.51 ± 0.03 vs 0.44 ± 0.3), PI (0.79 ± 0.08 vs 0.62 ± 0.06), PSV (20.7 ± 1.6 vs 21.8 ± 2.0 cm/s), TAMV (14.1 ± 1.3 vs 15.7 ± 1.7 cm/s), and plasma progesterone concentration (12.8 ± 1.2 vs 10.2 ± 1.2 ng/ml). The differences approached significance for the following end points: RI ($P < 0.07$), PI ($P < 0.07$), and progesterone concentrations ($P < 0.09$).

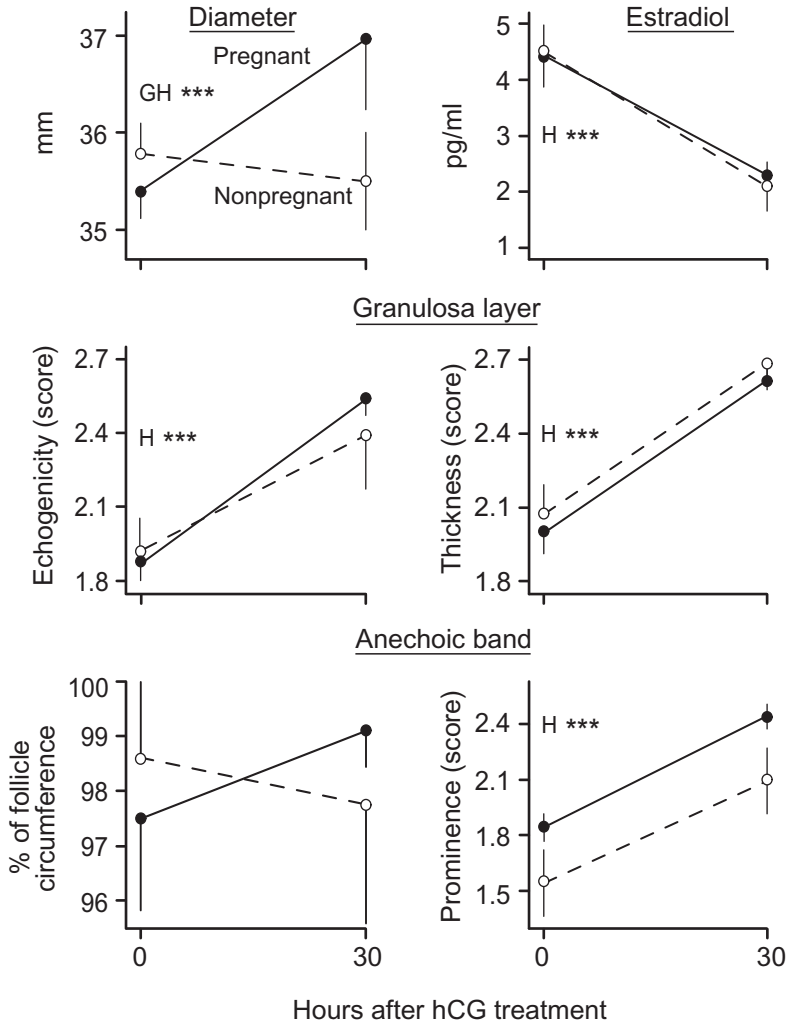

Figure 2. Values (mean ± SEM) for diameter of the preovulatory follicle, plasma estradiol concentrations, and B-mode characteristics of the follicle wall in mares that became pregnant (n = 14) or nonpregnant (n = 7). A single injection of hCG was given when the follicle was 34.0 to 37.0 mm (Hour 0), and mares were bred 30 h later (Hour 30). Scores are for minimal (1) to maximal (3) for echogenicity and thickness of the granulosa and prominence of the anechoic band. The asterisks indicate a significant main effect (H, hour) and significant interaction (GH, group by hour) as follows: *** $P < 0.001$.

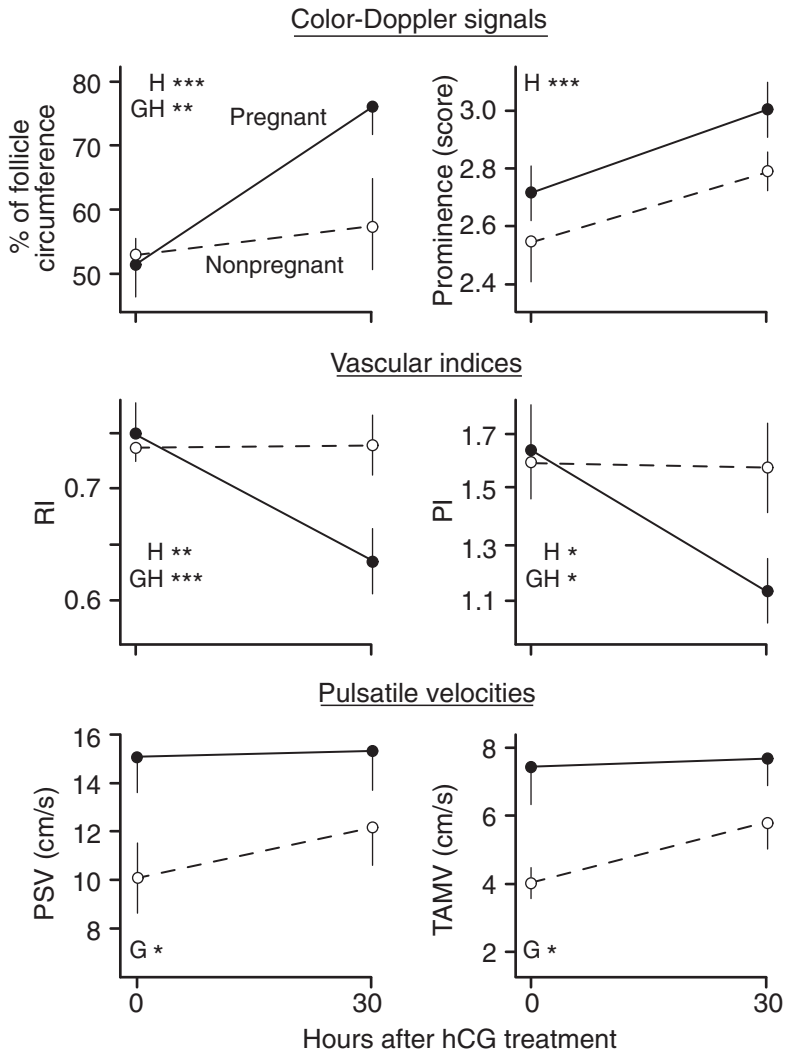

Figure 3. Values (mean ± SEM) end points obtained by Doppler ultrasonography for the preovulatory follicle in mares that became pregnant (n = 14) or nonpregnant (n = 7). A single injection of hCG was given when the follicle was 34.0 to 37.0 mm (Hour 0), and mares were bred 30 h later (Hour 30). Scores are for minimal (1) to maximal (4) for prominence of the color-Doppler signals. The indices (RI, PI) are inversely related to the extent of tissue vascular perfusion downstream from the point of assessment at the most prominent intraovarian color signal. RI = resistance index; PI = pulsatility index; PSV = peak systolic velocity; TAMV = time-averaged maximum velocity. The asterisks indicate significant main effects (G, group; H, hour) and significant interactions (GH) as follows: * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$).

Discussion

Results supported the hypothesis that a higher pregnancy rate is associated with greater blood flow to the preovulatory follicle. Support was indicated by the vascular changes between Hour 0 (hCG treatment) and Hour 30 (breeding), including a greater percentage of the follicle wall with blood-flow Doppler signals and a greater reduction in RI and PI of an intraovarian vessel in mares that became pregnant than in mares that did not. The RI and PI are indices from the spectral mode that are inversely related to the extent of vascular perfusion in the tissue downstream from the point of vessel assessment (Ginther and Utt, 2004; Zwiebel and Pellerito, 2005). The relative blood-flow velocities (PSV and TAMV) did not increase differentially in the

pregnant group between Hours 0 and 30 but were higher in the pregnant group at both hours. That is, blood velocity was already higher in the intraovarian vessel of the pregnant group by the time the follicle reached 34 to 37 mm (Hour 0) and remained elevated until just before breeding (Hour 30). Apparently, increased blood velocity in the ovary with the preovulatory follicle preceded the increased vascular perfusion of the follicle or tissue distal to the spectral evaluation. The positive association between establishment of pregnancy and the extent of vascularization of the preovulatory follicle is a novel finding, except for reports for women in oocyte and embryo transfer or assisted-reproduction programs (cited in Introduction).

An unexpected result was the differential increased diameter of the follicle between Hours 0 and 30 in the mares that became pregnant. Previous reports have indicated that the follicle does not increase in diameter during about 2 d before ovulation in both control and hCG-treated mares (Koskinen *et al.*, 1989; Gastal *et al.*, 2006). Previous studies, however, did not separate the mares into future pregnant and nonpregnant groups. The increased diameter in the present study in the pregnant group, therefore, does not contradict the previous findings. The diameter increase was < 1.0 mm in each of the seven mares in the nonpregnant group and > 1.0 mm in 57% of the 14 mares in the pregnant group. Further study will be needed to determine the practicality of using diameter increase as an indicator of the likelihood of future pregnancy in individual mares.

The B-mode echotextural characteristics averaged over Hours 0 and 30 were not different between the pregnant and nonpregnant groups. The main effect of hour indicated an increase in values between Hours 0 and 30 in both groups for echogenicity and thickness of the granulosa and prominence of the anechoic band. These results confirm those of the previous characterization study (Gastal *et al.*, 2006). The estradiol decrease following hCG treatment occurred in both groups and also confirms the previous results.

Results for the two stallions were combined and used in the statistical analyses, even though the pregnancy rate for Stallion A was higher than for Stallion B. Mares bred by Stallion A had a significantly lower RI and PI and greater follicle diameter 30 h after hCG treatment (before breeding) than for mares bred by Stallion B. Thus, despite the strict randomization of mares between stallions, Stallion A was assigned to a greater proportion of mares with prebreeding follicle characteristics that were shown in this experiment to be associated with establishment of pregnancy. When only Stallion B was considered, the significant differences between the pregnant and nonpregnant groups involved the same end points as for the combined stallions. Of the 10 mares assigned to Stallion A, the one mare that did not become pregnant had the lowest follicle blood-flow percentage, the highest RI and PI, and the lowest TAMV just before breeding. These considerations indicate that the stallion effect was not a confounding factor in the test of the hypothesis.

The mechanism associated with the detrimental effect of inadequate blood flow to the preovulatory follicle on oocyte competence or pregnancy rate is not known. Given that the vascular system delivers nutrients to tissues, the relationship between oocyte quality and blood flow may be similar, at least in part, to the relationship between oocyte maturation and nutrition. Dietary intake affects follicular-fluid concentrations of steroids and insulin-like growth factors and oocyte morphology (Callaghan *et al.*, 2000), but a direct relationship between follicle blood flow and these factors apparently has not been considered.

None of the luteal end points 7 d after ovulation indicated a difference in luteal morphology, vascularity, or function between pregnant and nonpregnant mares. The decrease in progesterone and increase in RI and PI in the nonpregnant group were equivocal in that the differences only approached significance, indicating a need for further study. In this regard, the literature also seems conflicting (Sevinga *et al.*, 1999), and a need for further study with greater number of subjects is indicated.

In conclusion, diameter of the preovulatory follicle increased in the pregnant group but not in the nonpregnant group between the time of hCG treatment and breeding 30 h later. Doppler ultrasonographic characteristics of the preovulatory follicle 30 h after hCG injection indicated that follicle blood flow was greater in mares that became pregnant than in mares that did not. Characteristics that supported this conclusion were percentage of circumference of the follicular wall with blood-flow signals and resistance and pulsatility indices taken at the most prominent intraovarian color signal.

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