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Relationships of changes in B-mode echotexture and colour-Doppler signals in the wall of the preovulatory follicle to changes in systemic oestradiol concentrations and the effects of human chorionic gonadotrophin in mares

E L Gastal, M O Gastal and O J Ginther

Eutheria Foundation, Cross Plains, Wisconsin 53528, USA

Correspondence should be addressed to O J Ginther, Animal Health and Biomedical Sciences, 1656 Linden Drive, University of Wisconsin, Madison, Wisconsin 53706, USA; Email: ginther@svm.vetmed.wisc.edu

Abstract

A duplex grey-scale and colour-Doppler ultrasound instrument was used to study the changes in the wall of the preovulatory follicle in mares. When the follicle reached ≥35 mm (hour 0), mares were randomized into control ($n = 16$) and human chorionic gonadotropin (hCG)-treated ($n = 16$) groups. The hCG treatment was given at hour 0. Scanning was done every 12 h until hour 36, every hour between hours 36 and 48, and every 12 h thereafter until ovulation. Blood was sampled every 12 h for oestradiol assay. During the period 0–24 h post-treatment, oestradiol concentrations decreased in the hCG group and increased in the controls (significant interaction). During the period 0–36 h post-treatment, thickness and echogenicity of the granulosa increased in the hCG group but not in the controls. During the period 36 to 12 h before ovulation, granulosa and colour-Doppler end-points increased in the control and hCG groups (hour effects), while oestradiol was decreasing in both groups. The prominence and percentage of follicle circumference with an anechoic band peripheral to the granulosa and colour-Doppler signals in the follicle wall, indicating arterial blood flow, decreased during the period 4 to 1 h before ovulation (hour effects). Results indicated that the ultrasonographic changes of the wall of the preovulatory follicle were not associated temporally with changes in oestradiol concentrations and prominence of an anechoic band, and colour-Doppler signals decreased during the few hours before ovulation. The hypothesis that the latter portion of the ovulatory LH surge has a negative effect on systemic oestradiol was supported by the immediate decrease in oestradiol concentrations when hCG was injected.

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Introduction

In mammals, a preovulatory follicle consists of a fluid-filled antrum encapsulated progressively (from inside to outside) by granulosa, theca interna and theca externa (Hunter 2003). The avascular granulosa is separated from the theca interna by a basement membrane. The theca interna is a layer of glandular cells mingled with a rich capillary plexus, and the theca externa is a more fibrous layer. The follicle develops a double wreath of blood vessels. The outer wreath consists of arterioles and venules in the theca externa, and the inner wreath consists of a prominent capillary network in the theca interna. Expansion of the theca vessels and capillaries involves formation of new vessels as well as dilation of existing vessels (Familiari *et al.* 1991). The microvasculature of follicles has been extensively reviewed and illustrated for several species (Kikuta *et al.* 1991). The preovulatory follicle is surrounded also by a two-tiered network of lymphatic vessels in humans (Jdanov 1969) and pigs (Andersen 1926). These vessels enlarge during the follicular phase. Although apparently not studied, it seems likely that similar wreaths of lymphatic vessels encapsulate the preovulatory follicle of horses.

Using transrectal ultrasonic B-mode (grey-scale) imaging in the mare, the granulosa is identifiable as an echoic band enclosing the antrum (Ginther 1995*a*). The two layers of theca can be assumed on the basis of position, but the boundary between the theca externa and interna is indistinct. The inner boundary of the theca interna can be located as a result of its close apposition to the basement membrane of the granulosa. However, the entire thickness of the wall reportedly can be

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estimated by differentiating the outer periphery of the theca externa from the surrounding stroma in women (Martinuk *et al.* 1992).

In the initial equine B-mode ultrasonographic study (Pierson & Ginther 1985), granulosa thickness increased as the interval to ovulation decreased. In another study (Carnevale 1998), mean scores for echogenicity and thickness of the granulosa increased during the 24 h before ovulation. In addition to an increase in echogenicity of the granulosa, prominence of an anechoic band in the expected area of the theca layers increased daily and progressively in the period 3 to 1 days before ovulation (Gastal *et al.* 1998). Based on similar end-points, days and interval (1 day) between scanning sessions as for the above study, it has been concluded that the slopes of the regression lines for these characteristics are related to impending ovulation (Chan *et al.* 2003). In another study (Carnevale *et al.* 2002), several mean pixel values increased in an approximately linear fashion during the 14 h before ovulation in human chorionic gonadotropin (hCG)-treated mares. To our knowledge, a comparison of ultrasonographic changes that presage natural versus hCG-induced ovulation has not been reported.

In women, a line of decreased reflectivity (Picker *et al.* 1983) or a double contour (Jaffe & Ben-Aderet 1984, Jaffe *et al.* 1987) has been described in the wall of the preovulatory follicle. This characteristic is apparently similar to the anechoic band described in mares (Gastal *et al.* 1998, Chan *et al.* 2003). Computer-assisted image analysis and mathematical modelling of the dynamic changes in the combined layers of the wall from stroma to antrum are being investigated in women and cattle (Singh *et al.* 2003), but results have not yet been directly compared with the anechoic band that was described in the earlier literature. However, the combined layers of the wall of the follicles that eventually ovulate are thicker and have lower pixel intensity values than follicles that are destined to become atretic in women (Martinuk *et al.* 1992) and cattle (Tom *et al.* 1998); an anechoic band would contribute to lower mean pixel intensity values.

Colour-Doppler ultrasound has been used extensively in women to evaluate the arterial blood flow in the wall of the preovulatory follicle. Colour-Doppler scanners respond to the movement and direction of flow of red cells in arteries and arterioles, and display blood flow in colour on the B-mode image (Ginther & Utt 2004). Doppler colour-flow mapping and spectral analyses (e.g. velocity, resistance index) have been done in women for different regions of the preovulatory follicle (Brannstrom *et al.* 1998) and during ovulation (Bourne *et al.* 1991). Other colour-Doppler studies involved the relationship between arterial spectral responses (Chien *et al.* 2004, Pan *et al.* 2004) or percentage of follicle circumference with colour signals (vascular perfusion; Bhal *et al.* 1999, Coulam *et al.* 1999) of the donor follicle and the viability of transferred embryos. Colour-Doppler ultrasound has been used for studying vascular perfusion of the dominant follicle in mares during the transition between the anovulatory and ovulatory seasons (Acosta *et al.* 2004), but did not include the preovulatory follicle.

In mares, circulating concentrations of oestradiol reach maximum about 2 days before ovulation (Ginther *et al.* 2005*a*). Concentrations in the follicular fluid have been reported to both decrease (Gérard *et al.* 1999) and not decrease (Watson & Sertich 1991) before ovulation. Concentrations of luteinizing hormone (LH) begin to increase at the end of the luteal phase and reach maximum the day after ovulation (Ginther *et al.* 2005*a*). The preovulatory oestradiol surge begins to decrease 3 days before the peak of a prolonged LH surge (about 7 days) in mares (Ginther *et al.* 2005*a*), contrasting with about 1 day before a short LH surge (<1 day) in many other species (e.g. cattle, dogs, goats, sheep; Pineda & Dooley 2003). The profile of the inclining portion of the ovulatory LH surge in mares is biphasic, consisting of an initial slow increase and a subsequent rapid increase that begins on the day oestradiol begins to decline (Ginther *et al.* 2005*a*,*b*). These temporal relationships indicate that the rapid increase in LH has a negative effect on systemic oestradiol concentrations in mares, but this assumption has not been investigated *in vivo*. *In vitro*, segments of follicular wall from preovulatory oestrus follicles are less responsive to stimulation of oestradiol by LH than segments taken from late dioestrus follicles (Sirois *et al.* 1990). These temporal relationships and the long interval between the oestradiol and LH peaks indicate that the mare may be a useful model for studying the effect of LH on oestradiol.

The temporal relationships of B-mode echotextural and colour-Doppler changes in the wall of the preovulatory follicle to systemic hormone concentrations have not been studied in any species. In this regard, oestradiol may be involved considering that it is produced by the developing follicles and has a pronounced effect on blood flow and oedema in other organs of the reproductive tract (Pineda & Dooley 2003). The large size of the preovulatory follicle, its accessibility for ultrasonography, and the profound echotextural and vascular structure (Gastal *et al.* 1998, Kerban *et al.* 1999) indicate that the mare would be a useful model for studying the role of oestradiol in the echotextural and vascular changes in the follicle wall.

The purpose of the present experiment was to compare B-mode with colour-Doppler imaging for characterizing the echotextural and blood-flow changes in the equine preovulatory follicle as ovulation approaches; and also to relate the changes in ultrasonographic structure to changes in oestradiol concentration. In addition, comparison was made between natural ovulations and hCG-induced ovulations. The inclusion of an hCG-treated group in the experiment provided an opportunity to examine the hypothesis that the increasing LH activity beginning 2 days before ovulation has a negative effect on oestradiol concentrations and accounts for the preovulatory decrease in systemic oestradiol.

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. Non-lactating pony mares ($n = 38$ oestrous cycles in 38 mares; 6–20 years old; 300–450 kg) of mixed breeds were used during September and October in the Northern Hemisphere (latitude, 43° N). The score for body condition for all mares was high throughout the experiment (score ≥7; Henneke *et al.* 1983). 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. The mares selected had a docile temperament and had no apparent abnormalities of the reproductive tract – as determined by ultrasound examinations (Ginther 1995*a*). Mares that developed codominant follicles (≥30 mm), haemorrhagic anovulatory follicles (Ginther 1995*a*) or slow follicle evacuation during the experiment were not used in the statistical analyses. Slow follicle evacuation during the ovulatory process was defined as evacuation of <75% of follicular fluid in ≥180 min.

Experimental groups

Mares with a growing 28 mm follicle 15 days after ovulation were scanned daily by B-mode ultrasonography until a ≥35 mm follicle was detected. When the largest follicle reached ≥35 mm (hour 0) and the score for endometrial echotexture was between 3 and 4 (oestrus-like; Ginther 1995*a*), mares were randomly assigned to control ($n = 16$) and hCG-treated ($n = 16$) groups. At hour 0, mares in the control and hCG groups received an i.v. injection of 1 ml saline or 1 ml saline containing 2500 IU of hCG respectively. Vials containing the saline and hCG treatments were colour coded by a third person, so that the two operators evaluating the follicles did not have knowledge of treatment. After hour 0, scanning was performed at 12-h intervals until hour 36. From 36–48 h, scanning was done every hour in both groups because 70% of ovulations in the hCG group were expected to occur during this time (Ginther 1992). For mares that did not ovulate by hour 48, scanning was done again every 12 h until ovulation.

In a separate impending-ovulation group, contemporary mares were scanned with B-mode ultrasonography every 24 h until six mares were found that were expected to ovulate within 12 h. Thereafter mares were scanned every hour, and all mares ovulated in 9 to 3 h. Impending ovulation was estimated by combinations of a thick and echogenic granulosa, decreased turgidity, loss of spherical shape, stigma, detached granulosa segments and echoic spots in the antrum (Pierson & Ginther 1985, Carnevale *et al.* 1988, Townson & Ginther 1989, Gastal *et al.* 1998). Turgidity was estimated from the extent of the shape change in response to gentle pressure from the transducer.

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 colour-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. Experimental data collection began when the largest follicle reached ≥35 mm. 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). In colour-Doppler mode, the degree and direction of flow in the arteries and arterioles are indicated by colour signals and the intensity of the colour is proportional to the velocity of blood flow (Ginther & Utt 2004). The colour mode was used to display the blood flow in vessels of the follicle wall; the spectral mode was not used. All Doppler scans were performed at a pulse-repetition frequency of 8 Hz, and constant colour-gain settings were used. The B-mode and colour-Doppler end-points were evaluated while the entire follicle was scanned in a slow continuous motion several times. Real-time B-mode/colour-Doppler images were captured with an on-line digital video-taping system and stored for selection of images for illustrative purposes.

End-points

Several B-mode end-points were quantified. Follicle diameter was obtained from the average of height and width of the antrum at the apparent maximal area from two frozen images. The anechoic layer was identified as a grey to black band, irregular in thickness, located in the expected area of the thecal layers (Figs 1–3). The percentage of the circumference of the follicle wall with an anechoic band was estimated from the 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 quantitative end-points (prominence of anechoic band, echogenicity and thickness of granulosa) were considered by scoring from 1 to 3 (minimal to maximal); intermediate scores at 0.1 increments were included. Scores were assigned without reference to the score from the previous examination. The prominence of the anechoic band was scored from grey and thin to black and thick as described previously (Gastal *et al.* 1998, 1999, Chan *et al.* 2003). The granulosa was identified on the basis of its position next to the antrum (Fig. 1), and its echogenicity was scored from hypoechoic (grey) to hyperechoic (white), according to Carnevale (1998) and Gastal *et al.* (1998, 1999). The thickness of the granulosa was scored from thin to thick, as described previously (Carnevale 1998, Gastal *et al.* 1999). The evaluations from the real-time images encompassed the entire follicle and minimized the

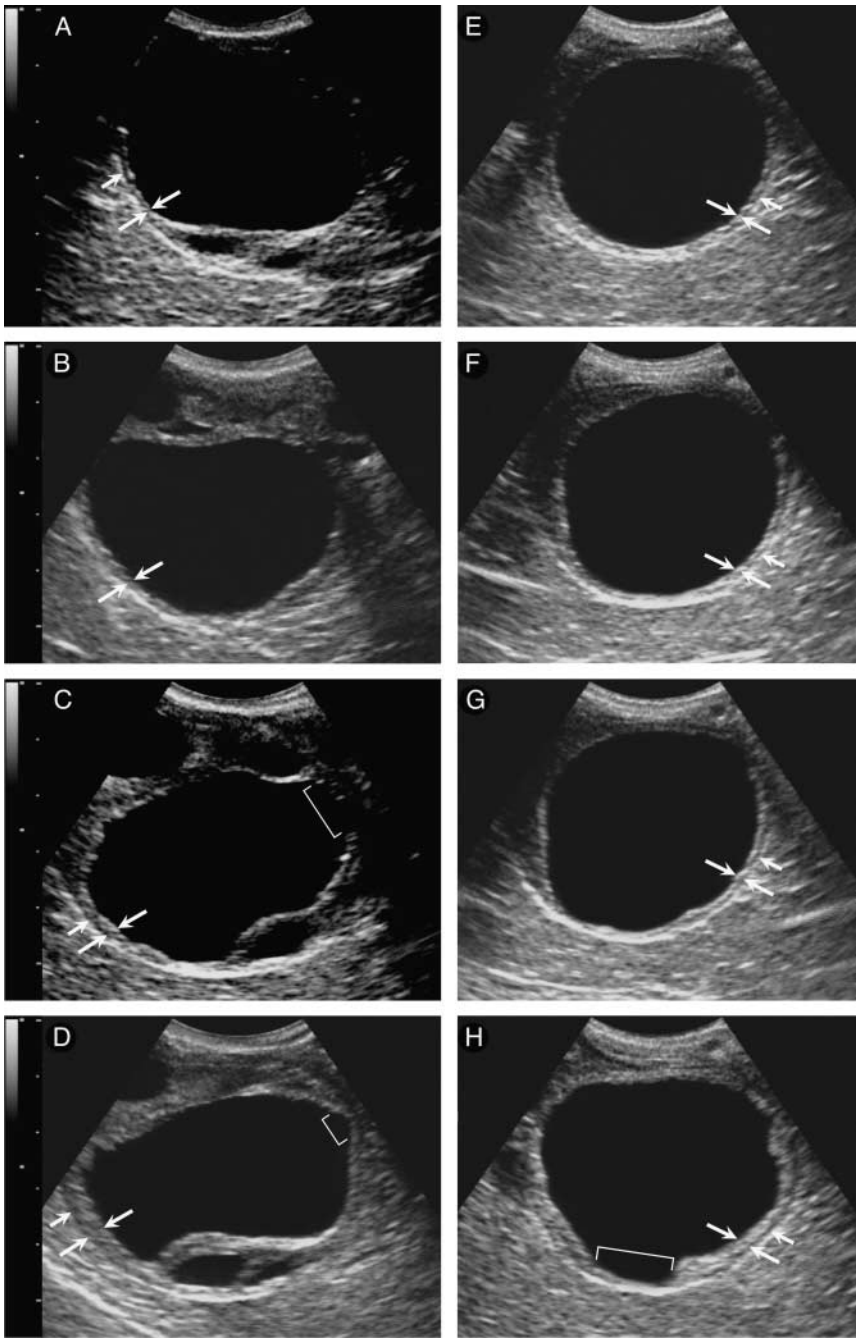

Figure 1 Sequential B-mode ultrasonograms of preovulatory follicles of two mares at different hours after the largest follicle reached ≥35 mm (hour 0, treatment time). In the sonograms in the left column, follicles of a control mare are shown at hours 0 (A), 12 (B), 36 (C) and 40 (D). Ovulation was detected at hour 41. In the right column, follicles of an hCG-treated mare are shown at hours 0 (E), 12 (F), 24 (G) and 37 (H) after treatment at hour 0. Ovulation was detected at hour 39. As ovulation approached (top to bottom) for each mare, the granulosa (large opposing arrows) became thicker. Segments of the follicular wall with an anechoic band (single short arrows) are shown for both mares. Note the decreased thickness of the granulosa at the apical area (square brackets), when compared with the thicker opposite granulosa. Distance between graduation marks (left) is 1 cm.

impact of localized areas of the follicle wall that were obscured by ultrasound artifacts (Ginther 1995*a*). The validity of the scoring approach has been documented for both B-mode (Ginther 1995*a*) and colour-Doppler mode (Ginther & Utt 2004) ultrasonography and had the advantage of assessing the entire follicle rapidly and thereby minimized animal discomfort.

For colour Doppler-mode, the percentage of circumference of the follicle wall with an apparent network of arterioles was estimated from the blood-flow colour displays (Figs 2 and 3) of the real-time sequential two-dimensional planes of the entire follicle. A similar approach has been used in women, although it is not clear whether a single selected image or the entire real-time scan was evaluated (Chui *et al.* 1997, Bhal *et al.* 1999, Coulam *et al.* 1999). In the present experiment, the transducer was held at various angles to get the maximum overall colour signal throughout the circumference of the follicular wall; the angle between the ultrasound beam and arterial flow affects the detectability and extent of the colour signals (Zwiebel & Pellerito 2005). The prominence of the colour displays was based on intensity and thickness, and was

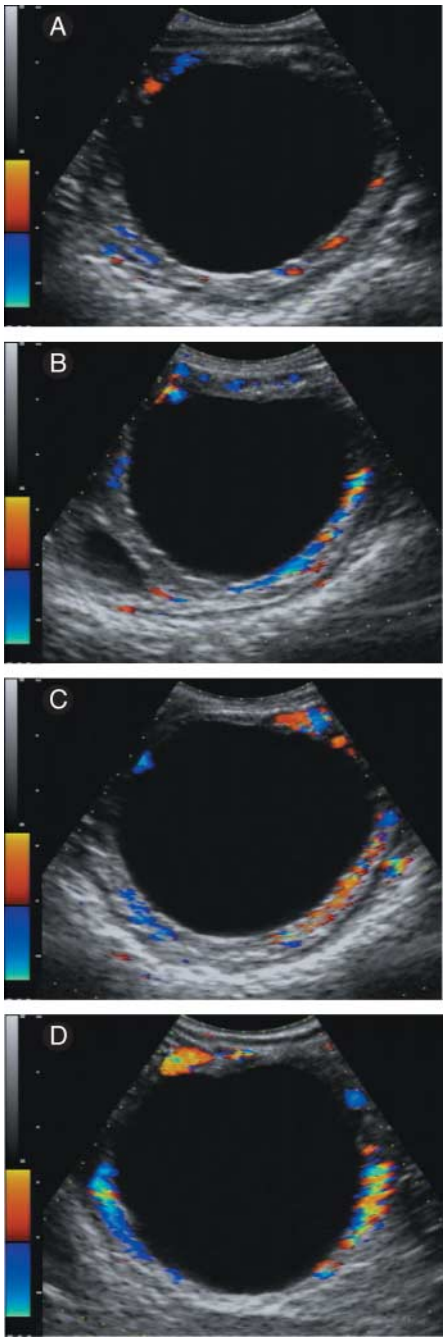

Figure 2 Sequential colour-Doppler ultrasonograms of preovulatory follicles of a control mare at 36 (A), 38 (B), 44 (C) and 60 h (D) after the largest follicle reached ≥35 mm (hour 0, treatment time). Ovulation was detected at hour 84. Note the increase in colour-Doppler signals (amount of colour dots and intensity) as the follicle matured (top to bottom). Most of the colour-Doppler signals are located within or extend beyond the width of the anechoic band of the follicle wall. Distance between graduation marks (left) is 1 cm.

scored from 1 to 4 (minimal to maximal). These two end-points were quantified subjectively and independently by two experienced operators during scanning by one of the operators. The percentage of wall with colour-Doppler displays and the prominence of the colour displays did not differ between operators (data not shown), and the means for the two operators were used in the statistical analyses.

The ultrasonic echotexture of the endometrium of the control and hCG-treated groups was scored from 1 to 4 (minimal to maximal), based on the extent of anechoic areas of the endometrial folds (Ginther & Pierson 1984). The echotexture was used as an indicator of oestrogenic stimulation.

Blood samples and oestradiol assay

Jugular blood samples were collected into heparinized tubes in control and hCG-treated groups. Samples were centrifuged (1500 ***g*** for 10 min), decanted and stored (−20 °C) until assay. Collections were done every 12 h from the detection of a ≥35 mm follicle until ovulation. Systemic oestradiol concentrations were assayed for hours 0, 12, 24 and 36 of the post-treatment period and at 36, 24, 12 and 0 h before the post-treatment ovulation. Plasma concentrations of oestradiol were measured by a double-antibody radioimmunoassay kit (Double Antibody Estradiol; Diagnostic Products Corporation, Los Angeles, CA, USA), as described and validated for mare plasma (Ginther *et al.* 2005*a*). The intra- and interassay coefficients of variation were 4.5 and 12.6% respectively, and sensitivity was 0.1 pg oestradiol/ml.

Statistical analyses

Data were not normally distributed for any quantitative end-point, according to Kolmogorov–Smirnov tests (Zar 1984). Therefore, a ranking procedure (Conover 1999) was used for the diameter of the dominant follicle and scores for the quantitative end-points (thickness and echogenicity of the granulosa, prominence of the anechoic band and prominence of colour-Doppler signals). The percentage of the circumference of the wall with an anechoic band or a colour display, and the percentage change in oestradiol concentrations, were analyzed after arcsine transformation. Percentages were used for oestradiol during the post-treatment period (hours 0–36) because of a disparity ($P < 0.05$) between groups at hour 0; it was thought that the disparity could contribute to significant differences at later hours. Data were analyzed by the SAS MIXED procedure to determine the main effects of group and hour, and their interaction, using a repeated statement to account for the autocorrelation between measurements (version 8.2; SAS Institute Inc., Cary, NC, USA). Unpaired *t* tests were used to locate differences between groups and paired *t* tests were used between hours within a group when a significant main effect or an interaction was obtained. A probability of $P < 0.05$ indicated that a difference was significant. Data are presented as the mean ± S.E.M., unless otherwise indicated.

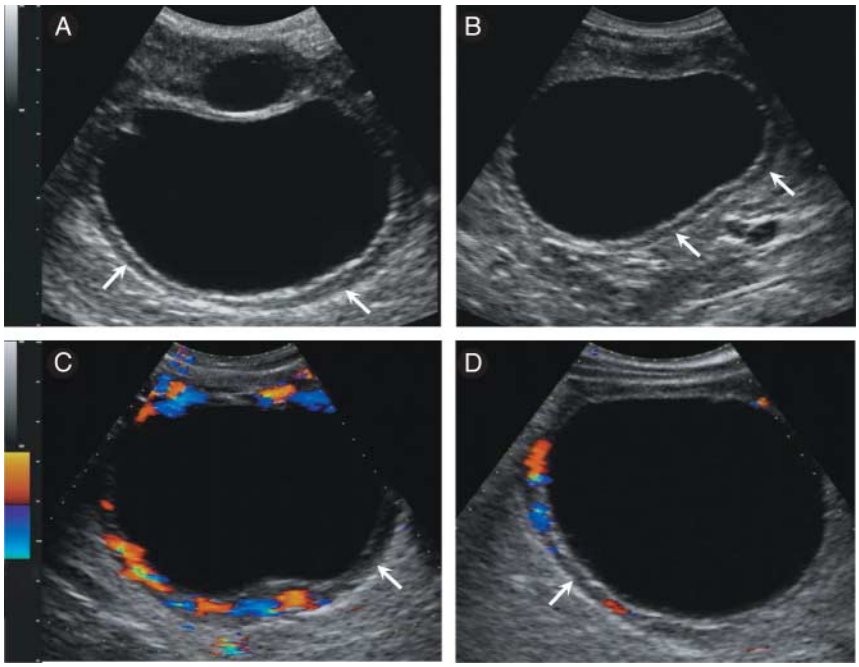

Figure 3 Selected B-mode (A and B) and colour-Doppler (C and D) ultrasonograms of preovulatory follicles of four control mares. (A and B) Ultrasonograms at 2 days after the largest follicle reached ≥35 mm (treatment time) and 1 day before ovulation. (C) Ultrasonogram at 4 days after the largest follicle reached ≥35 mm and 2 days before ovulation. (D) Ultrasonogram at 4 days after the largest follicle reached ≥35 mm and 3 h before ovulation. Images in panels A and D were magnified to improve visibility of the anechoic band. Note the anechoic band (arrows) and colour-Doppler signals located within or extending beyond the width of the anechoic band. Distance between graduation marks (left) is 1 cm.

Results

Mares were removed from the analyses because of haemorrhagic anovulatory follicles in the control ($n=2$) and impending-ovulation ($n=1$) groups and slow evacuation of follicular fluid during ovulation ($n=3$) in the hCG group. After removal of these mares, totals of 14, 13 and 5 mares were available for statistical analyses in the control, hCG and impending-ovulation groups respectively. The five mares in the impending-ovulation group were combined with the two control mares that ovulated during the period from 36 to 48 h, so that hourly examinations preceding ovulation were available in seven mares that were not treated with hCG. These seven non-hCG-treated mares were compared with seven hCG-treated mares that ovulated during 36–48 h. The time required for each follicle evaluation was 1–2 min and 1–3 min for B-mode and colour-Doppler mode ultrasound scanning respectively. The interval from first appearance of a follicle ≥35 mm to ovulation was longer ($P<0.0001$) in the saline-treated group (78.4 ± 6.3 h; range, 41–132 h) than in the hCG-treated group (36.0 ± 2.1 h; range, 12–41 h).

Means and significant main effects and interactions for the quantitative B-mode (Fig. 4) and colour-Doppler (Fig. 5) end-points are shown for the post-treatment period (0–36 h), the preovulatory period (36 to 12 h before ovulation) and the impending period (4 to 1 h before ovulation). During the post-treatment period, diameter increased in the controls but not in the hCG-treated group, whereas a mean increase in thickness and echogenicity of the granulosa occurred only in the hCG-treated group, as indicated by hour effects within each group and the group-by-hour interactions (Fig. 4). Only the hour effect was significant for prominence of the anechoic band, and percentage and prominence of the colour-Doppler display (Figs 4 and 5). During the preovulatory period, the follicle was larger in controls than in the hCG group (group effect). Thickness and echogenicity of the granulosa during the preovulatory period increased with no difference between groups and no interaction effect. There were no significant differences for prominence of the anechoic band, but the hour effect was significant for percentage of circumference with a band. The hour effect was significant for prominence and percentage of circumference with colour-Doppler display, and the group effect was significant for percentage of circumference. During hourly examinations for the impending period averaged over the hCG- and non-hCG- treated mares, an increase over hour (hour effect) occurred for granulosa thickness and echogenicity, a decrease for prominence and percentage of circumference of the anechoic band, and a decrease for both the Doppler end-points. The only other effects within the preovulatory and impending-ovulation periods was an interaction in the impending period for prominence of the anechoic band.

The group-by-hour interaction for endometrial score was significant ($P<0.005$) for hours 0 to 36 of the post-treatment period (Fig. 6). The interaction was attributable to lower scores in the hCG group at hours 24 ($P<0.009$) and 36 ($P<0.009$). The profiles for endometrial score for 36 to 0 h before the post-treatment ovulation were similar to the scores for hours 0–36 of the post-treatment period (data not shown). For the percentage change in oestradiol concentrations over hours 0–36 of the post-treatment period, both main effects and the interaction were significant ($P<0.01$; Fig. 6). Percentage change increased ($P<0.0005$) by hour 24 in the controls and decreased

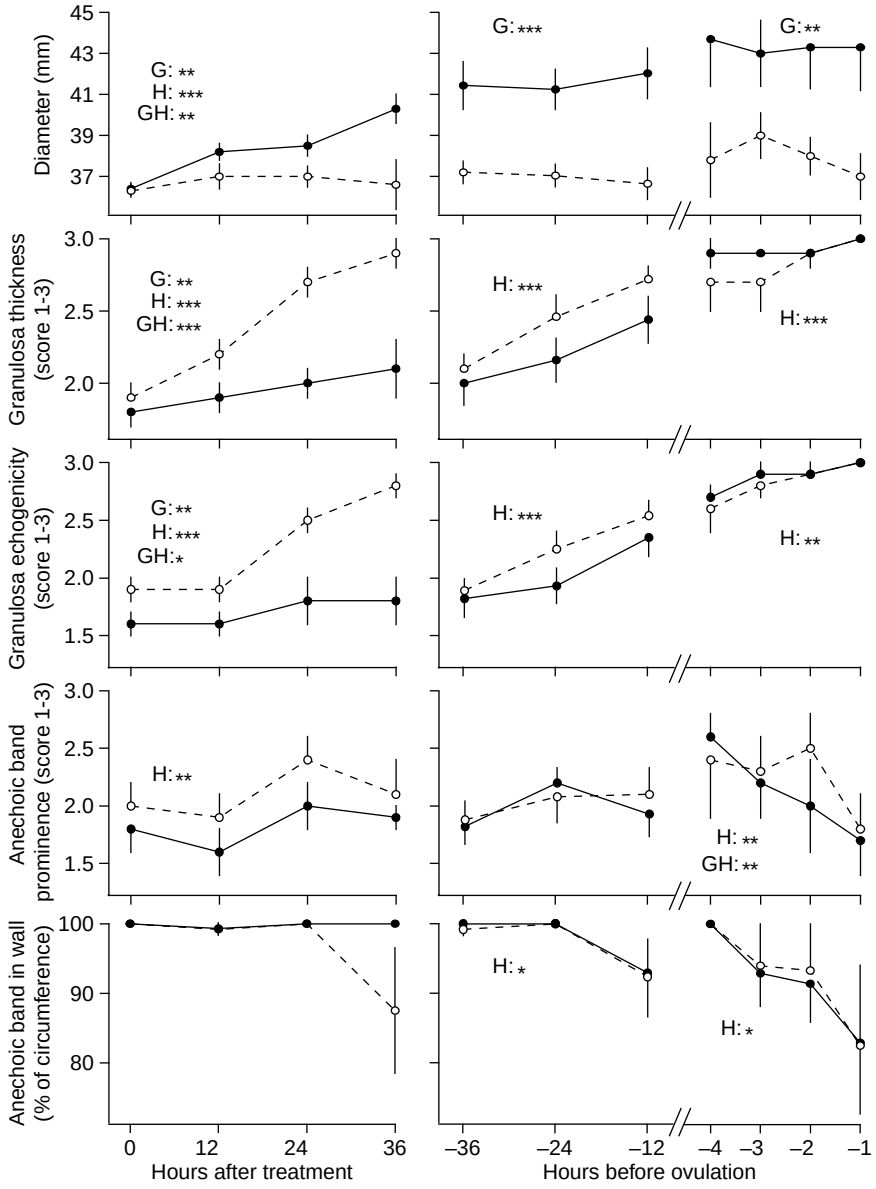

Figure 4 Mean ± S.E.M. for B-mode ultrasound end-points for the wall of the preovulatory follicle in control (●) and hCG-treated (○) groups during the post-treatment period (hours 0–36; $n = 8$–14), the preovulatory period (hours −36 to −12; $n = 8$–14) and the impending-ovulation period (hours −4 to −1; $n = 4$–8). The asterisks indicate significant main effects (G, group; H, hour; GH, interaction) as follows: $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$.

($P < 0.0001$) over hours 0–36 in the hCG-treated group. For oestradiol concentrations during the 36 to 12 h before ovulation, both main effects ($P < 0.02$) and the interaction ($P < 0.008$) were significant (Fig. 7). Concentrations decreased as ovulation approached; the interaction was attributable to lower concentrations in the hCG group at 12 ($P < 0.02$) and 0 h ($P < 0.04$) before ovulation.

Discussion

The absence of a mean diameter increase during the 36 h before ovulation in the controls is consistent with a previous report (Koskinen *et al.* 1989) that mean diameter increased until 2 days before ovulation and remained constant thereafter. During the preovulatory period when mean diameter was no longer increasing in the controls, mean oestradiol concentrations were decreasing. This relationship also extended to the hCG group after treatment; diameter ceased to increase and oestradiol concentrations began to decrease within 12 h after the hCG injection. Apparently, this is the first report in mares of cessation of follicle growth and a decrease in oestradiol beginning immediately after hCG treatment.

The hypothesis of a negative effect of LH on oestradiol during the late preovulatory period was supported by the immediate decrease in oestradiol concentrations when hCG was injected. The negative preovulatory effect of LH on oestradiol production is consistent with the report that hCG administration in mares resulted in an intrafollicular increase in 17β-hydroxysteroid dehydrogenases (Brown *et al.* 2004); these enzymes have the potential of reducing circulatory concentrations of estradiol associated with the

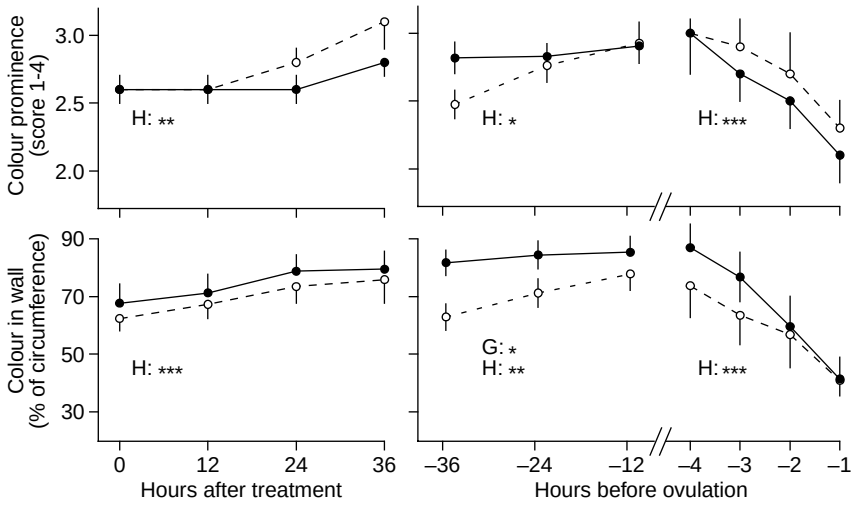

Figure 5 Mean± S.E.M. for colour-Doppler end-points for the wall of the preovulatory follicle in control (●) and hCG-treated (○) groups during the post-treatment period (hours 0–36; $n = 8–14$), the preovulatory period (hours −36 to −12; $n = 8–14$) and the impending-ovulation period (hours −4 to −1; $n = 4–8$). The asterisks indicate significant main effects of hour (H) or group (G) as follows: $*P < 0.05$; $**P < 0.01$; $***P < 0.001$. There were no GH interactions.

LH surge. Furthermore, granulosa cells from mature dominant equine follicles, as opposed to growing follicles, had lower levels of the following steroidogenic enzymes: steroidogenic acute regulatory protein, P450-side chain cleavage, 3β-hydroxysteroid dehydrogenase and aromatase (Belin *et al.* 2000). In rats, the degradation of aromatase mRNA by the LH surge has been demonstrated (Fitzpatrick *et al.* 1997). A recent study (Ndiaye *et al.* 2005) in cattle indicated that treatment with hCG was associated with a decrease in the expression of mRNA for aromatase enzyme in granulosa cells of dominant follicles and a decrease in the oestradiol:progesterone ratio in follicular fluid. Thus, the hCG in the present study may have blocked oestradiol production by down-regulation of aromatase and other steroidogenic enzymes. The reason for the lower oestradiol concentrations 12 h before ovulation in the hCG group compared with the controls is unknown, but speculatively may have reflected greater LH-like activity of the hCG dose than for endogenous LH in controls. In addition, a reduction in the number of granulosa cells due to cessation of cellular divisions (Robker & Richards 1998) and a difference between hCG and LH in the interaction with LH receptors (Roess *et al.* 1997) may have played a role.

Increasing accumulation of fluid in the endometrial folds (increase in endometrial scores) during oestrus is indicated by the presence of anechoic areas on B-mode images and is used in the management of mares for

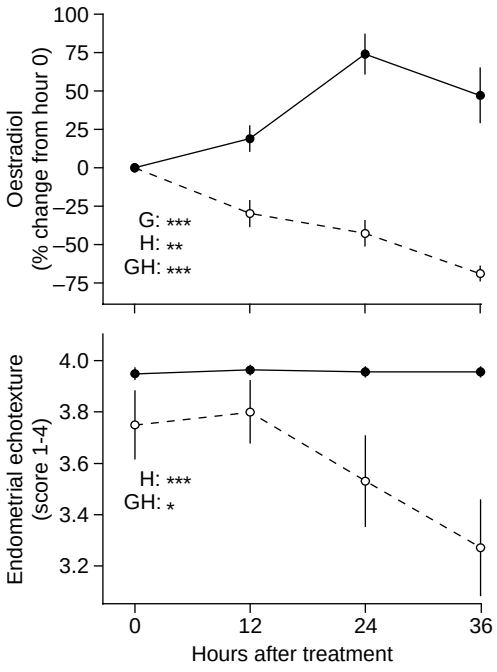

Figure 6 Mean± S.E.M. for percentage change in systemic oestradiol concentrations relative to hour 0 and scores for endometrial echotexture in control (●) and hCG-treated (○) groups during the post-treatment period (hours 0–36; $n = 8–14$). The asterisks indicate significant main effects (G, group; H, hour; GH, interaction). Probabilities are as follows: $*P < 0.05$; $**P < 0.01$; $***P < 0.001$.

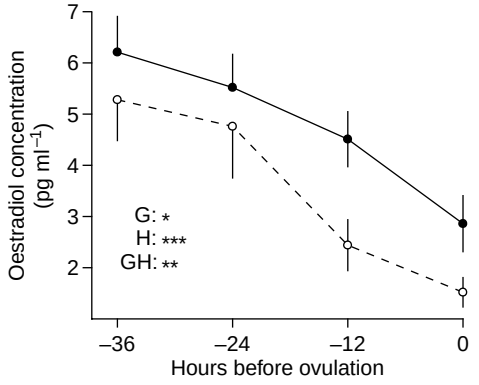

Figure 7 Mean± S.E.M. for systemic concentrations of oestradiol in control (●) and hCG-treated (○) groups during the preovulatory period (−36 to 0 h relative to ovulation; $n = 12–14$). Time of treatment of the hCG group was equivalent on average to −36 h. The asterisks indicate significant main effects (G, group; H, hour; GH, interaction). Probabilities are as follows: $*P < 0.05$; $**P < 0.01$; $***P < 0.008$.

breeding (Ginther & Pierson 1984). In the present study, the decrease in endometrial scores after hCG treatment is attributable on a temporal basis to the decrease in systemic oestradiol and appeared to begin about 12 h after the decrease in oestradiol in the hCG group. This interpretation is consistent with reports of a decrease in scores for both endometrial echotexture and oestrous behaviour beginning on the day before ovulation (Hayes *et al.* 1985), and similarly for endometrial scores and circulating oestradiol concentrations (Pycock *et al.* 1995).

During hours 0–36 post-treatment, an increase in B-mode scores for thickness and echogenicity of the granulosa was detected for the hCG-treated group, but not for the control group, and accounted for the group-by-hour interactions. These two granulosa characteristics, however, were not different between groups during the preovulatory and impending-ovulation periods, indicating that the effects on the granulosa during the 36 h before ovulation were similar for natural and induced ovulations. The post-treatment change in the hCG-treated mares presumably represented preovulatory changes, as indicated by a mean 36-h interval from treatment to ovulation. These ultrasonic changes in the granulosa have been previously been reported for non-hCG-treated mares (see Introduction). The changes in thickness and echogenicity of the granulosa were not attributable temporally to oestradiol; in both groups the scores for both granulosa end-points increased during the period 36 to 12 h before ovulation, while oestradiol concentrations were decreasing (compare Figs 4 and 7).

The increase in thickness and echogenicity of the granulosa beginning 36 h before ovulation is compatible with the results of histological studies in mares. Expansion of the granulosa doubled between 0 and 39 h after hCG treatment and was attributed to a pronounced accumulation of mucosubstances between the cells (Kerban *et al.* 1999). The expansion was first detected at 12 h after hCG treatment in agreement with the increase in thickness and echogenicity in the present ultrasound results. The increased echogenicity of the granulosa probably resulted from separation between cells by the mucosubstances, which would be expected to increase the reflective quality of the resulting tissue interfaces (Ginther 1995*b*).

The increase in prominence of the anechoic band in the control and hCG groups after the follicle reached ≥35 mm has been reported previously for non-hCG-treated mares (Gastal *et al.* 1998, Chan *et al.* 2003). The percentage of follicle circumference with an anechoic band did not change in either the control or hCG groups during the post-treatment period and during 36 and 24 h before ovulation (preovulatory period). Over these periods, the anechoic band involved most of the circumference, agreeing with results of previous studies with non-hCG-treated mares (Gastal *et al.* 1998, 1999). The anechoic band reached about 95% of the circumference when the future dominant follicle reached about 22 mm (Gastal *et al.* 1999). During the post-treatment period, the changes in the anechoic band were not associated temporally with changes in oestradiol concentrations; oestradiol increased in the controls and decreased in the hCG group, whereas prominence of the band increased slightly, averaged over the two groups. During the 4 h before ovulation, the prominence and percentage of circumference for the anechoic band decreased in both the control and hCG groups. The decrease in circumference with an anechoic band between 24 and 12 h before ovulation represented a decrease in two mares in each group from 100% to a mean of 50%. The two mares in the hCG group ovulated 1 and 2 h after the last examination at 12-h intervals, accounting for the detection of a circumference decrease at the last examination. Similar information was not available for the control mares. The decrease in prominence and circumference of the anechoic band during the few hours before ovulation is a new finding; the previous preovulatory study (Gastal *et al.* 1998) did not involve hourly assessments.

Histologically, the fluid between cells in the follicle wall is enclosed by both vascular capillaries and terminal lymphatics and probably accounts at least partially for the anechoic band. In this regard, the capillary and lymphatic systems originate in a network of terminal sacs (Detweiler 1993), and considerable dilation of capillaries in the theca has been described as ovulation approaches in mares (Kerban *et al.* 1999) and women (Govan & Black 1981). In addition, the ovulatory process has been described as an inflammatory reaction (Espey & Lipner 1994), including hyperaemia, congestion, increased vascular permeability and oedema (Kerban *et al.* 1999). On this basis, the fluid of the anechoic band also could be attributed to oedema or an excessive accumulation of fluid in the intercellular spaces (Jones & Hunt 1983). However, the anechoic band increases in prominence (Gastal *et al.* 1999) long before the inflammatory oedema associated with ovulation in mares (Kerban *et al.* 1999) and, in the present study, the prominence decreased during the few hours before ovulation. In summary, histological reports in mares (Kerban *et al.* 1999) and women (Govan & Black 1981) do not adequately clarify the nature of the anechoic band seen *in vivo* by ultrasonic imaging. In this regard, a layer consisting primarily of fluid would be altered during excision and formalin fixation.

The changes in colour-Doppler prominence did not differ between the controls and hCG-treated mares during the post-treatment period, similar to changes in prominence of the anechoic band but unlike the difference between groups for granulosa echotexture. During the preovulatory period (36 to 12 h before ovulation), the difference between groups for percentage of wall with colour displays may have represented a more rapid change in the hCG group, owing to the shorter interval from a 35 mm follicle to ovulation. In this regard, the two groups were similar during the 12 h before ovulation. The most striking effect was the decrease in percentage of follicle circumference with colour display and the decrease in the prominence scores for colour during the last 4 h before

ovulation, similar to the decrease in circumference and prominence for the anechoic band. The prominence of the colour-Doppler displays, as well as thickness of the granulosa, was greater at the base than at the apex of follicles that had lost their spherical shape. Colour-Doppler displays were not detected in the apical area of the follicle. This result is consistent with decreased circumference with colour signals when considering the entire circumference in the present study in mares and a previously reported (Brannstrom *et al.* 1998) blood flow decrease in the thin apical area of the follicle as ovulation approaches in women. The colour-Doppler signals of blood flow were dispersed within the anechoic band (Figs 2 and 3), indicating that the anechoic band included the fluid of blood vessels, presumably venules as well as arterioles. In this regard, some of the colour-flow signals often extended beyond the anechoic band and even appeared to involve the granulosa. This may have involved, at least in part, an artifact wherein the signal extends beyond the vessel walls (Zwiebel & Pellerito 2005). Considering that the blood vessels are expected to be in the theca externa and that the anechoic band is in close apposition to the granulosa, it is likely that the anechoic band involved both the theca externa and theca interna. As with the echotextural changes, there was no temporal indication that the colour-Doppler displays were related to the positive effects of oestradiol on the vasculature.

In conclusion, hCG treatment given when the preovulatory follicle was $\geq$35 mm was associated with immediate cessation of growth of the follicle and decreased systemic oestradiol. The oestradiol decrease supported the hypothesis of a negative effect of LH on oestradiol concentrations during the late preovulatory period. B-mode scores for thickness and echogenicity of the granulosa and colour-Doppler end-points increased during the preovulatory period while oestradiol was decreasing in both control and hCG-treated mares, indicating a lack of temporality with oestradiol changes. Based on reported histology, the increased echogenicity of the granulosa was attributed to an expected increase in reflective tissue interfaces, resulting from separation of cells by a mucoid intercellular coating. The prominence and percentage of the follicle circumference of both an anechoic band peripheral to the granulosa and colour-Doppler signals, indicating blood flow, decreased during the 4-h period before ovulation. Based on reported locations of arterioles, venules, capillaries and lymphatic vessels, the anechoic band was interpreted to involve both thecal layers.

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