Transrectal Tonometric Measurement of Follicular Softening and Computer Assisted Ultrasound Image Analysis of Follicular Wall Echotexture During Estrus in Mares

Natalie D. Bragg, DVM, Roger A. Pierson, PhD, Dylan G. Buss, DVM, and Claire E. Card, DVM, PhD

A drop in follicular pressure detected by transrectal tonometry and ultrasonographic detection of separation of the layers of the follicular wall indicates impending ovulation in both natural and hormonally manipulated estrus. In early estrus, the administration of hCG accelerates follicular growth and the onset of follicular softening, but does not induce an increase in follicular wall thickness. Authors’ Addresses: Canadian Food Inspection Agency, Government of Canada, Edmonton, Alberta (Bragg); Department of Large Animal Clinical Sciences, Western College of Veterinary Medicine (Buss, Card); and Department of Obstetrics and Gynecology, Royal University Hospital (Pierson), University of Saskatchewan, Saskatoon, SK, Canada S7N 5B4. © 2001 AAEP.

1. Introduction
The equine preovulatory follicle undergoes a series of changes in preparation for ovulation: an increase in size, change in follicular shape, tone, wall thickness, and wall echotexture.1–7 Reproductive management of mares for assisted reproductive techniques (ART), such as artificial insemination (AI), commonly includes the administration of prostaglandin (PG) to induce estrus, and human choriionic gonadotropin (hCG) to time ovulation. During cycles where AI of cooled transported semen is planned, a single periovulatory insemination is often used because of higher costs, restricted availability, and potentially reduced viability of processed semen compared to fresh semen. However, ovulation within 48 h of hCG administration occurs only about 85% of the time, and therefore there is a need to further improve or predict the response to treatment. Promising new investigative tools such as transrectal follicular tonometry and computer-assisted image analysis of follicular attributes have been used to provide quantitative information concerning the physiologic status of a follicle. Computer-assisted image analysis of ultrasound attributes is an important technological advancement because when properly implemented, it is precise and repeatable.

The objectives of the study were to determine if measurement of follicular softening using a transrectal tonometric device and computer-assisted ultrasound image analysis of follicular attributes including follicular wall echotexture could be used to describe physiological changes in natural estrus, and to determine how hormonal manipulation (PG
and hCG) influenced these parameters. We also investigated their use as ancillary tools to predict the time of ovulation.

2. Materials and Methods

The reproductive tracts of 10 young light horse mares were examined via a transrectal ultrasonography every 12 h during estrus for 4 successive cycles: cycle 1 natural estrus; cycle 2 PG-induced estrus; cycle 3 PG-induced estrus with hCG given when size of the dominant follicle >35 mm; and cycle 4 PG-induced estrus with hCG given when grade II endometrial edema was obtained. The antrum of the dominant follicle was measured and the degree (0 no edema, IV maximal edema) of endometrial edema recorded. Data were retrospectively aligned from the day of ovulation (time zero). Preovulatory follicular tone was quantitated using a novel tonometric device which was validated using abattoir specimens and was designed to objectively measure pressure in relative PSI units. Cross-sectional real-time and still-frame images of the preovulatory follicular wall were captured on S-VHS videotape, subsequently digitized and analyzed using a customized computer program according to the method of Pierson et al using spot metering analysis.

Spot measuring entailed the placement of 5 mm² circles in the base of the follicular wall to sample pixels. The pixels within the circular spots were assigned a numeric value by the computer, which corresponded to the grey-scale of each pixel. The pixel values within a spot were summed and expressed in terms of a mean numeric pixel value (NPV) and a corresponding standard deviation. An overall mean was calculated by summing and averaging the NPV in 4 spots.

Linear time-series analysis employed a line which was drawn through a section of the ovary (parenchyma, follicular wall, antrum) and the pixel intensities along the line were plotted. The pixel intensities (expressed as NPV) reflected the amplitude of the echos along the line. A plot of the number of pixels (length) and pixel intensities was made. The area under the region of the plotted curve which corresponded to the parenchyma, follicular wall and antrum was identified by the change in pixel intensity. The area under the region of the curve that represented the follicular wall was compared over time in estrus.

Regional surface analysis involved the placement of a computer generated grid onto the selected area of a follicle. The image system then generated a 3-dimensional output with length and width expressed in millimeters and height in pixel intensity. A color-enhanced, three-dimensional image was produced which showed the contour of the follicular wall. The features of the follicular image were assessed visually.

Data were analyzed at p < 0.05 using a repeated measures ANOVA (duration of estrus) on follicular wall NPV by time from ovulation. Post-hoc comparisons between days before ovulation were performed using the Bonferroni method. Fischer’s Exact Test was used to compare the percentages of mares that had changes in follicular size, a drop in follicular pressure, and separation in the follicular wall by group 24 hours before ovulation.

3. Results

A summary of the significant results are presented in Table 1. Data shown are mean ± SEM. The duration of estrus was shortened from 6.0 ± 0.5 days to 2.0 ± 0.5 days in cycles where hCG was administered (p < 0.05). Follicular tonometric readings fluctuated throughout the duration of estrus and decreased 0.5–1.0 PSI between 24 and 12 h prior to ovulation in each of the 4 cycles (p1 = 0.01; p2 = 0.01; p3 < 0.001; p4 = 0.03) (subscripts denote cycle number). Follicular pressure (PSI) at the time of ovulation was similar among the 4 cycles (2.35 ± 0.15, 2.65 ± 0.12, 2.52 ± 0.10, 2.60 ± 0.08, respectively). Follicular antral diameters (mm) increased up to 24 h before ovulation and were: 45.2 ± 5.3 (p1 = 0.01); 46.9 ± 13.5 (p2 = 0.03); and 43.0 ± 7.8 (p4 = 0.02), respectively. Follicular growth rates (mm/day) for the 4 cycles up to 24 h before ovulation were 2.5 ± 0.6, 1.9 ± 0.5, 1.7 ± 0.1 and 4.0 ± 0.1, respectively. One mare in cycle 2 ovulated an exceptionally large follicle (78 mm). Mean NPV of the follicular wall increased during estrus until ovulation for cycles 2: 3.1 ± 1.9 (p2 = 0.05); 3: 11.7 ± 5.3 (p3 < 0.001); and 4: 10.7 ± 4.3 (p4 = 0.03). There was a significant progressive increase during estrus in the follicular wall thickness as assessed by linear time series analysis (area) in cycle 1: 71.3 ± 24.6 (p1 = 0.005) and cycle 2: 127 ± 45.3 (p2 = 0.01). Separation of the layers in the base of the dominant follicle was noted in a total of 32/40 (80%) of cycles using regional contour analysis. There were no differences between groups in the frequency distribution of follicular size, drop in

| Table 1. Frequency Distribution of Selected Follicular Parameters 24 Hours Before Ovulation in 10 Estrous Mares in Natural Estrus (Cycle 1), PG-Induced Estrus (Cycle 2), PG + hCG when a Dominant Follicle (F) F > 35 (Cycle 3), and PG + hCG when Endometrial Edema is Grade II (Cycle 4) |
|---------------------------------|---|---|---|---|
| F size at ovulation             | 1 | 2 | 3 | 4 |
| P 30–35                        | 1 | 1 | 0 | 1 |
| P 36–40                        | 4 | 3 | 5 | 5 |
| P 41–45                        | 5 | 4 | 5 | 4 |
| P > 46                         | 0 | 2 | 0 | 0 |
| ≤0.5 Drop in PSI               | 7 | 8 | 8 | 9 |
| wall separation                | 7 | 8 | 8 | 9 |
PSI, or wall separation in the 24 h before ovulation using Fischer’s Exact Test.

In the 5 cycles that mares that did not ovulate within 48 h of hCG treatment the preovulatory change that was most often lacking was a change in the thickness of the follicular wall.

4. Discussion

The parameters listed in Table 1 were chosen because they showed significant changes over time during estrus in all 4 cycles and included: follicular size, drop in follicular pressure, and separation of the layers in the follicular wall, but there were no significant differences in these parameters between groups in the 24 hours before ovulation. This was not the anticipated outcome. Following hCG treatment in early estrus when small firm follicles were present, we predicted that there would be no change in follicular pressure prior to ovulation. The data indicate that hCG speeds follicular maturation through a process that accelerates growth, induces steroidogenic enzymes, and creates tonometric and histomorphologic changes, such as separation, in the follicular wall.

Computer assisted image analysis provided a quantitative tool to monitor changes in echotexture of the preovulatory follicular wall. Increases in mean NPV of the follicular wall in cycles 2–4 indicated that the brightness of the pixel elements increased in cycles with hormonal manipulation. These changes were subtle and represented increases of 3.1–5.1 NPV over time. The echotextural change may be related to an increase in the acid mucosubstances secreted by the granulosa cells in cycles where hCG is used.

Linear time series analysis allowed a precise quantification of the thickness of the base of the follicular wall by analyzing the pixels and showed that small increases in follicular wall thickness were present in natural and PG induced cycles but not in hCG treated cycles. A histologic study of changes in follicular wall thickness showed an increase in wall thickness of 50 μm in estrous mares treated with hCG when they had a 35 mm follicle.10 This small increase is below the precision of the equipment used in the study. The present and historical data support our finding of no detectable increase in follicular wall thickness following hCG using ultrasonography, hence thickening of the follicular wall would not be a useful indicator of a response to hCG. There was one report of an increase in follicular wall thickness, following the administration of hCG in estrous mares.3 The difference between this study and that report may relate to the timing of hCG administration, because administration in mid to late estrus may coincide with the normal physiologic increase in LH, and addition of hCG may further enhance this process.

Separation of the granulosa and thecal cell layers was apparent in many follicles using ultrasonography and regional contour analysis. An increase in blood vessels, edema, hemorrhage, and hyperemia has been reported in between the theca and granulosa cell layers in mares near ovulation that were treated with hCG.10 These changes are believed to correspond to the separation noted in the follicular wall. Separation in the follicular wall was noted at the same time as the drop in follicular pressure. Maturational changes in the theca and granulosa cell layers may influence the secretion or processing of follicular fluid into the antrum of the follicle. The data indicate that the follicular wall does not change appreciably in thickness. The thinning of the wall of the dominant follicle reported by transrectal palpation may relate to a detection of the separation of the layers of the granulosa and theca layers, rather than a stretching of the wall by an increase in intrafollicular pressure.

Application of tonometry and computer assisted image analysis is limited primarily by the custom design of both the tonometry device (patent pending) and patented software (Pierson). Equipment costs for the tonometry device were under $1500 and the computer acquisition and image analysis equipment $10,000. The examination process with the tonometry device was rapid and took less than 3 min to obtain the readings. The only skill required for use of the tonometry equipment was proficiency in ovarian palpation. With further refinement, the tonometry equipment could easily be incorporated into the housing or scan head of an ultrasound probe. The use of tonometry allowed more objective quantification of the changes in follicular tone or pressure than rectal palpation. The drop in follicular pressure was best detected with serial measurements and was significant 12–24 h before ovulation, however just prior to ovulation follicular pressure was variable, with some follicles becoming firmer and others softer.

Acquisition of the ultrasound image for analysis was as rapid as freezing an image for measurement. The image analysis process of retrieving and evaluating the echotextural features of an image required about 5 min per image to complete.

Predictions concerning the future of ultrasound imaging include a convergence with digital computer technology. Digital imaging has already allowed the easy storage, movement, and analysis of images from an ultrasound unit to a computer and the internet. Many ultrasound machines currently incorporate software to estimate gestational age and so on, and predictably other advanced software features will be added as well. Computer-assisted image analysis has been used to determine the physiologic status of the corpus luteum, dominant follicle, and testis in other species. Further refinements and improvements in imaging and computer technology will lead to the ability to determine information about the physiologic status of tissues through echotextural analysis.

In conclusion, attainment of a dominant follicle with an antrum >35 mm, separation of the follicular wall, or wall separation in the 24 h before ovulation using Fischer’s Exact Test, would not be a useful indicator of a response to hCG.
wall layers, and a drop in follicular pressure are useful indicators of impending ovulation in natural and hormonally manipulated estrous cycles. These changes are believed to be linked to maturational changes in the theca and granulosa cell layers, which are accelerated by the administration of hCG in early estrus.

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References and Footnotes

*aAusonics Impact, 6mHz microconvex probe, Universal Ultrasound, Bedford Hills, NY 10507.
*bLutalyse, 5 mg, SQ, UpJohn Pharmacia, Orangeville, Ontario L9W 3T3.
*dSynergyne 1 ©, Dr. Roger Pierson, Royal University Hospital, College of Medicine, University of Saskatchewan, Saskatoon, SK Canada S7N.
*eStatistical Package for the Social Sciences (SPSS), Version 9.0, Chicago, IL.