Development of a subjective scoring system for evaluating corpus luteum function in beef cattle

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Abstract

The objective of this project was to develop a scoring system to evaluate blood flow through the corpora lutea and circulating progesterone concentrations in heifers and cows through the progression of a normal, nonmanipulated estrous cycle. Sixteen, non-lactating, beef females were subjected to reproductive tract ultrasound scans every other day for the duration of an entire normal bovine estrous cycle (22 days). Ovarian structures (follicles and corpora lutea) were identified and measured. Color Doppler mode was used to visualize blood perfusion through luteal tissue and was given a subjective score based on percent of the corpus luteum that showed color (0, no color; 1, small color at periphery; 2, up to 80% color; 3, >80% color). Venous blood samples were collected at the time of ultrasound examination, and serum progesterone values were used to confirm corpus luteum function. The area of the corpus luteum was correlated with serum progesterone concentrations (P <0.0001, r=0.45). Doppler score was strongly correlated with serum progesterone concentrations (P < 0.0001, r=0.62) and area of the corpus luteum (adjusted for lacunas) (P <0.0001, r=0.48). Peak serum progesterone concentrations were not correlated with age (P=0.74), weight (P=0.80), or BCS (P=0.13). There was a tendency for peak progesterone concentration to be correlated with the area of the corpus luteum (P=0.07, r=0.46). Doppler score was not correlated (P = 0.59) with ultrasonographer. In summary, Doppler scores can be utilized as an estimation of corpus luteum function, irrespective of scanning technician, and may be a more reliable measure than by corpus luteum area determination, alone.

Keywords: Bovine, ultrasound, progesterone, Doppler score, corpus luteum, perfusion

Introduction

Food animal veterinary medicine has embraced ultrasound technology, and the use of this diagnostic tool is continuing to rapidly expand in both private practice, and well as in research applications. Many of the more advanced capabilities of ultrasound were initially cost prohibitive, such that the addition of these capabilities were restricted by economics to research or referral institutions. As with most technological advancements, costs of adding additional capabilities to ultrasound units have decreased to approach potential value as an added diagnostic tool or veterinary practice service, if a routine application would exist. Doppler ultrasonography has been used extensively in cardiac evaluation for many years, but more recently, blood flow and perfusion of tissues of the reproductive tract has gained the attention of some researchers. More and more research trials are published, in which Doppler (color flow and power) is used to attempt to further elucidate normal processes, as well as potentially illustrate physiologic responses to treatments. Capturing Doppler images on portable ultrasound units is a very quick process that requires very little additional training for the skilled ultrasonographer. The practical implementation and interpretation of these images remains a challenge.

Progesterone and corpus luteum size

There is no doubt that adequate circulating progesterone (P_4) is an integral component to the reproductive success of the bovine. Higher progesterone in circulation has positive influences on the developmental competence of the oocyte, enhances conceptus development, and is critical for the establishment and maintenance of pregnancy.[1] Pregnancy in the cow has many direct and indirect economic benefits,[2] and attempts to maximize the opportunities to establish pregnancies are incessant. Many studies have shown a positive correlation between corpus luteum (CL) size and circulating progesterone concentrations.[3-11] As the CL is developing (days 0-7 of the estrous cycle), progesterone concentration increases proportionally to CL growth, nearly doubling between Day 4 and Day 7 of the cycle.[4] However, the CL will increase in size during the static phase (Day 7 to Day 14) by only

approximately 25 percent, but progesterone concentrations will double, again, during this period of time.[12] This is believed to be due to the angiogenic potential of the CL and the development of capillaries within the luteal tissue which permit greater delivery of blood (and signaling molecules) to the luteal tissue, as well as increased transportation of progesterone (and other signaling molecules) from the CL.[13] After exposure to prostaglandin $F_{2\alpha}$ (PGF), both progesterone and CL size decrease, but not in a proportional manner. Within 48 hrs of PGF administration, circulating progesterone levels decreased dramatically (80% decline in 48 hrs).[4,14] In contrast, the CL size decreased more gradually, with negative growth pattern (regression phase) similar in change and duration as the positive growth pattern (growth phase), rather than exhibit a dramatic decrease in size, as progesterone levels would suggest.[4,15] This finding supports the theory that CL perfusion is impacted in a much more immediate manner, by the decrease in blood volume circulating through the luteal tissue, as well as an increase in the resistance to blood perfusion through the luteal tissue.

Using Doppler as an estimate of CL function

Researchers in a previously mentioned study evaluated luteal blood flow in conjunction with CL size and progesterone concentrations.[4] Blood flow was estimated using image capture and PixelFlux software (version 1.0, Chameleon Software, Leipzig, Germany). Not surprisingly, luteal blood flow changes mirrored that of progesterone, but not CL size. Indeed, all three measures (CL size, luteal blood flow, and progesterone concentration) all increased in a linear fashion, nearly doubling from Day 4 to Day 7 of the cycle. As CL growth slowed during the static phase, luteal blood flow continued to increase comparable to progesterone (doubling, again, from Day 7 to Day 14).[4] This finding suggests that there may be opportunities to utilize Doppler technology to more accurately classify a CL as functional or non-functional, rather than using CL size to estimate function. It has been suggested that a luteal blood flow measure of $>0.6cm^2$ could be a reliable indicator for CL function.[4] In contrast to this suggested standard, findings of a study by Scully, et al,[9] did not observe as dramatic and divergent blood flow change between pregnant and nonpregnant beef heifers on day 18 after insemination, but rather concluded that the CL blood flow ratio (percent of the CL area with color pixilation) was increased in pregnant heifers on day 18, and could be more diagnostic of CL function. Current quantification systems are not yet available on smaller, more portable cow-side ultrasound units, which have greatly limited the applicability of this tool in an on-farm setting. As acceptance of Doppler technology for CL assessment becomes a staple of the bovine reproductive examination, if can be assumed that the manufacturers of the ultrasound units would add such applications as standard features to their machines. Until quantitative evaluation systems are available as software applications on the smaller, more widely used ultrasound machines, an alternative would be to use a subjective score to classify blood flow through the CL.[3,17]

A functional CL is critical to the success of the transfer of embryos, and a subjective visual assessment system may identify some recipients that would not be suitable candidates for transfer, if Doppler scoring indicates that CL perfusion is suboptimal. In addition, some researchers are attempting to utilize CL blood flow as a very early indicator of pregnancy status (day 16-20 after estrus),[3,7-9,17] which is especially valuable in the identification of pregnant and non-pregnant dairy cows, so that appropriate interventions can be implemented in a timely manner (prior to the onset of the next standing estrus).[3,17-19] Likewise, the greatest expense in an embryo transfer program is recipient costs, and the early identification of pregnant and non-pregnant recipients can aid in the economic management of the herd.[8] Some researchers have shown reliability and repeatability in open versus not open diagnoses.[3,7,8] In one study, the positive predictive value of pregnancy was 65.1%, and the negative predictive value was excellent at 98.5%, with accuracy of 74.8%.[7] These results were assessed very near the time of onset of the next estrus (day 20), and utilized subjective evaluations of CL perfusion.

The objective of this project to correlate serum P_4 concentrations with a subjective visual Doppler score, to be used as a chute-side assessment of CL function. Such a scoring system has potential utility in many bovine reproductive capacities.

Materials and methods

All procedures and sample collections were performed in accordance with the guidelines of the Purdue Animal Care and Use Committee. Seven non-pregnant nulliparous, five non-pregnant primiparous, and four non-pregnant multiparous *Bos taurus* (Angus X Simmental) females (all non-lactating) were housed in a dry-lot and were fed to maintenance, according to NRC recommendations. Body weight and body condition score (BCS) were recorded for all cows at the start of the study. All females were subjected to reproductive ultrasound examination (MicroMax, FUJIFILM Sonosite Inc, Bothell, WA) at a frequency of 7.5 MHz by one of three technicians every other day for the duration of one complete bovine estrous cycle. Ovarian structures (follicles, corpora lutea) were mapped and measured. If a corpus luteum (CL) was present, a color-flow Doppler sonography subjective score was recorded by the technician on a scale from 0-3 based on the following criteria: 0) no Doppler color; 1) small Doppler color on periphery of CL; 2) up to 80% of CL having Doppler color; and 3) greater than 80% of CL having Doppler color (Fig. 1).

Blood samples were collected by jugular venipuncture in 10 ml evacuated tubes (Vacutainer, Becton, Dickinson and Company, Franklin Lakes, NJ) on the day of ultrasound examination, and were placed on ice immediately after collection. Samples were cooled for 12-24 hours, and were centrifuged at 2800 x g for 20 minutes at 4°C, and serum was removed and stored at -20^{O}C until assayed. Plasma progesterone concentration was determined using an in vitro diagnostic kit; Siemens 06603261 Immulite® Progesterone Kit (Siemens Medical Solutions Diagnostics, Los Angeles, CA). To validate the progesterone assay for bovine plasma, parallelism, recovery and repeatability were checked. Dilution curves of plasma samples and pure progesterone standards were parallel. When known amounts of progesterone were added to bovine plasma, recovery rates ranged from 89.5% to 104%. Eight plasma samples were evaluated 10 times each and the intra-assay CV was 5.8%, indicating good repeatability. The inter-assay CVs for a known range of 0.52-1.26 mg/dL was 12.81, 5.99-11.1 mg/dL was 5.33 17.1-30.5 mg/dL was 7.79.

Correlation analyses were conducted using the Spearman correlation coefficients in Proc Corr of SAS version 9.3 (SAS Inst. Inc., Cary, NC). Variables analyzed were Doppler score (DOP), progesterone serum concentrations (PROG), area of the CL (AREA), and day of ultrasound (DAY). Additional correlations between peak serum progesterone (the highest recorded progesterone concentration during the cycle) concentration and age (heifers, two year-olds and 3+ years), weight, BCS and area of the CL were performed. Correlation between Doppler score and ultrasound technician were evaluated. Any P-value less than 0.05 was considered significant. P-values >0.05 and ≤0.10 were considered a tendency.

Results

The area of the CL was correlated with serum progesterone concentrations (P <0.0001, r=0.45). Peak serum progesterone concentrations were not correlated with age (P=0.74), weight (P=0.80), or BCS (P=0.13). There was a tendency for peak progesterone concentration to be correlated with the area of the CL (P=0.07, r=0.46).

Doppler score was strongly correlated with serum progesterone concentrations (P < 0.0001, r=0.62). Doppler score was also significantly correlated with area of the CL (adjusted for lacunas) (P <0.0001, r=0.48). Ultrasonographer was not correlated with Doppler score (P = 0.59).

Discussion

Data from this project support the historical standards of circulating progesterone when determining cyclicity status,[20-22] and this information is validated by the visible presence of luteal tissue as well as the presence of adequate CL blood perfusion.

Circulating progesterone levels were well correlated with blood flow through the corpus luteum based on ultrasonographic evaluation of the CL utilizing color Doppler as a means of subjectively evaluating blood flow. Scores assigned to CL blood flow were repeatable between scanning individuals, and repeatability implies a degree of reliability, or consistency across individuals in the evaluation of CL function. This technique can therefore be utilized to assess the function (progesterone production) of a

corpus luteum. Assessment, or scoring, of CL function may serve a practical purpose, by providing an additional piece of information in a fertility examination, and presenting a clearer picture to the clinician of a given animals cyclicity status.

A corpus luteum can persist in the ovary of a cow for an extended period of time, physical destruction of the luteal tissue is a function of the immune system, and is a process that occurs over time.[13,23,24] However, decrease in progesterone concentrations subsequent to PGF exposure occurs at a much more accelerated rate, thereby indicating that the drop in progesterone is not simply due to luteal tissue destruction and removal. Power Doppler evaluation of the dynamics of blood flow through the corpus luteum before and immediately after PGF administration has shown that after an initial increase in CL blood flow (30 min to 2 hr after PGF administration), there was a significant decrease in vessel diameter and increased resistance to blood flow through the CL, effecting a dramatic decrease in the concentration of circulating progesterone in a very short period of time.[14] In contrast, in a study by Herzog et al,[4] the findings did not detect an increase in blood flow in response to PGF exposure. However, due to the short duration of the blood flow increase (30-120 minutes) it is possible to have missed this event. From a practical standpoint, if this blood flow increase is present during a single Doppler evaluation, it may be impossible to correctly differentiate between a functioning CL and one that is in the early stages of luteolysis.

Conclusion

Utilization of Doppler ultrasonography presents a very real opportunity for practitioners to make rapid, cow-side judgments regarding the function of the CL in the bovine female. However, the routine, practical use of this technology requires further exploration. From a purely observational standpoint, this study is in agreement with others, illustrating that CL function can be assessed by visualization of blood perfusion, and that these subjective assessments are repeatable between ultrasonographers. Ultrasound examination, utilizing B mode (presence or absence of a CL) and Doppler (color flow or power) using the proposed scoring system, with refinement and further large-scale studies, may provide a more rapid and less invasive means to categorize cows as having >1ng/ml of circulating progesterone (cycling) or <1 ng/ml of circulating progesterone (not cycling), than by venipuncture and serum progesterone assays.

The Doppler scoring system may aid in identification of “open” versus “not open” cows prior to early pregnancy diagnosis. Alternatively, and perhaps just as importantly, this scoring system may provide a means to estimate circulating progesterone concentrations at the initiation of an estrus and ovulation synchronization program, in an effort to maximize the developmental competence of the oocyte to be ovulated from a manipulated ovulation.

Each pregnancy in a bovine animal is an investment, and the value of a potential pregnancy is not one that a client is willing to put at risk without very significant evidence supporting an open diagnosis. Shortcomings still exist in our ability to differentiate between a functioning CL and one that is undergoing the very early stages of luteolysis. Given the conflicting reports regarding the immediate changes to CL blood flow, more work needs to be done to identify more objective means of quantifying blood flow and perfusion (CL function), as well as appropriate timing of evaluation of function, if we are to identify and institute early interventions (16-20 days after estrus) in the presumably open cow. Some of the larger, more costly, and less portable ultrasound machines have the capability to provide real-time quantifications of blood perfusion based on Doppler color. However, these machines are too valuable and cumbersome to be brought to the chute or the headlocks for use in routine bovine reproductive practice. Another significant challenge of quantifying CL blood flow is that often ovarian vessels not associated with the active CL may show color in the scan box or area, and may therefore give falsely elevated perfusion estimates. Many of the more recent publications site the use of image capture and importation of images to image analysis software to report quantification of blood flow through luteal tissues.[1,4,6,9] This software allows for estimation perfusion of only the area and structure of interest (CL) by allowing the user to establish a perimeter within which pixilation would be assessed. Although effective, this process does not allow the practitioner an immediate quantitative result on which treatment and management decisions can be based. The ultimate goal will be to develop quantitative values as a reliable

indication of the continuation or termination of the lifespan of a CL in the case of pregnancy or lack of pregnancy, respectively, within a timeframe (day 18-20) that would be convenient for incorporation of an additional ultrasound scan in the normal, routine reproductive management of cattle (once weekly examinations). With the increased durability and portability of other computational tools (i.e. iPads, tablets, laptop computers, etc), there may be an immediate opportunity to bring image analysis software to the barns where the cows are evaluated, if the ultrasound unit itself does not have image analysis software installed. Our group is in the process of developing more objective and quantitative parameters for the assessment of the function of ovarian structures in order to produce an immediate cowside result by utilizing a combination of the aforementioned technologies.

Conflict of interest

The authors declare no conflicts of interest.

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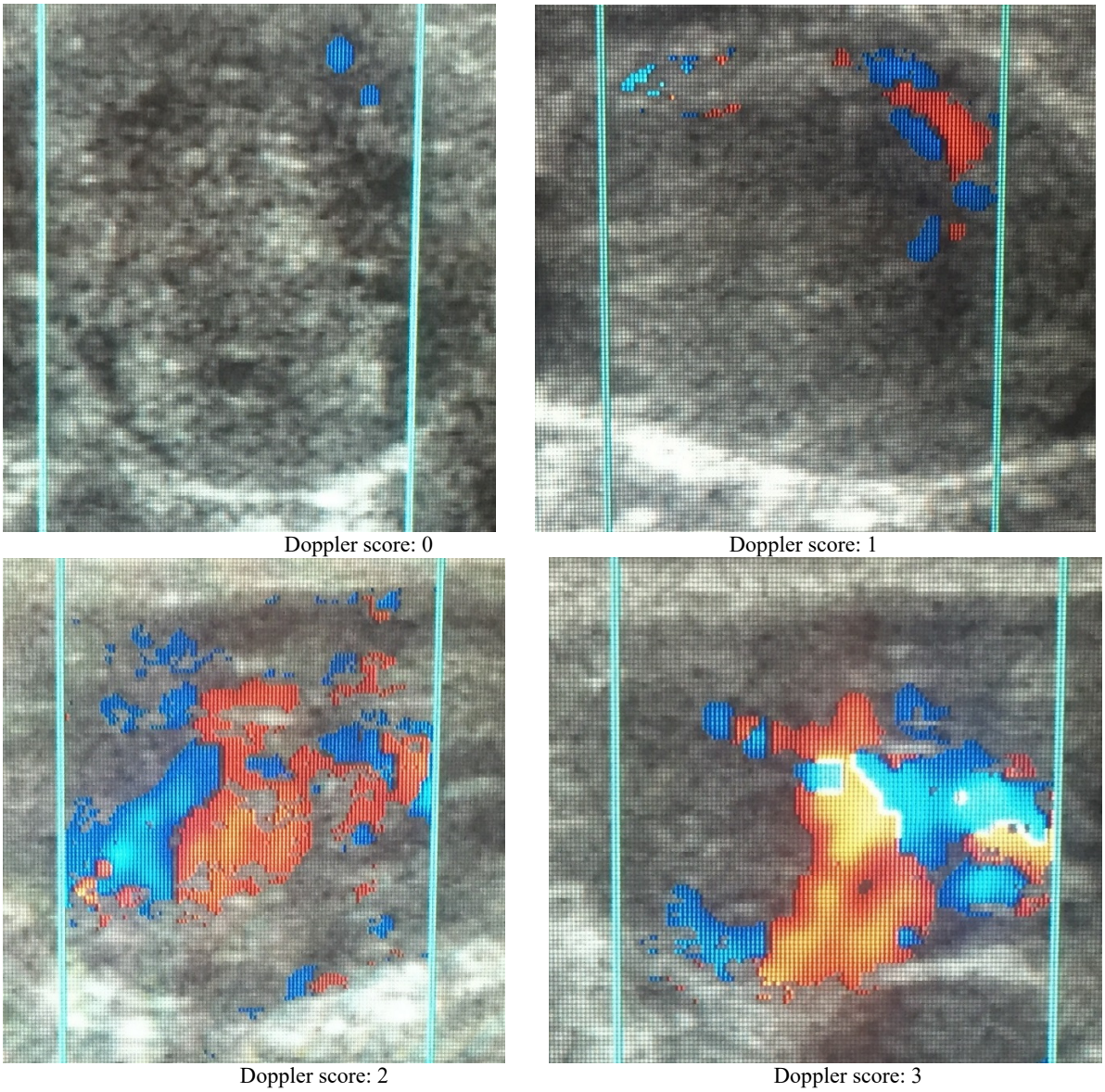

Fig. 1. Visual scoring of the level of perfusion of the CL (0: no perfusion, 1: low, 2: medium, 3: high)

(Editor's note: Photographs in this manuscript are available in color in the online edition of Clinical Theriogenology.)